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(54) Title: IMMUNOGENIC COMPOSITIONS COMPRISING LIVER STAGE MALARIAL ANTIGENS

(57) Abstract: A vaccine composition comprising a Th1-inducing adjuvant in combination with a protecting Liver Stage Antigen or immunological fragment thereof of a human malaria parasite, especially *Plasmodium falciparum*, with the proviso that when the immunological fragment is an immunological fragment of LSA-3 the Th1-inducing adjuvant is not Montanide. In one preferred aspect the Th1-inducing adjuvant comprises QS21, De-O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein the oil in water emulsion has the following composition: a metabolisable oil, such a squalene, alpha tocopherol and tween 80. In a further preferred aspect the protecting Liver Stage Antigen is Liver Stage Antigen 3 (LSA-3) or an immunological fragment thereof. A multivalent vaccine composition is also provided comprising the vaccine composition of the invention and in addition at least one other protecting antigen or an immunological fragment thereof, of a malaria parasite.

Immunogenic compositions comprising Liver Stage Malarial Antigens

The present invention relates to novel vaccine formulations, to methods of their production and to their use in medicine. In particular, the present invention relates to
5 a malaria antigen known as Liver Stage Antigen 3, or an immunological fragment thereof, in association with a Th-1 inducing adjuvant such as an oil in water emulsion or a vesicular adjuvant formulation comprising cholesterol, a saponin and optionally a lipopolysaccharide derivative. These and other aspects of the invention are described hereinbelow.

10

It has long been known that enterobacterial lipopolysaccharide (LPS) is a potent stimulator of the immune system, although its use in adjuvants has been curtailed by its toxic effects. A non-toxic derivative of LPS, monophosphoryl lipid A (MPL), produced by removal of the core carbohydrate group and the phosphate from the
15 reducing-end glucosamine, has been described by Ribi et al (1986, Immunology and Immunopharmacology of bacterial endotoxins, Plenum Publ. Corp., NY, p407-419).

A further detoxified version of MPL results from the removal of the acyl chain from the 3-position of the disaccharide backbone, and is called 3-O-Deacylated
20 monophosphoryl lipid A (3D-MPL). 3 De-O-acylated monophosphoryl lipid A is known from GB2 220 211 (Ribi). Chemically it is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains and is manufactured by Ribi Immunochem Montana. GB 2122204B also discloses the preparation of diphosphoryl lipid A, and 3-O-deacylated variants thereof. Other purified and synthetic
25 lipopolysaccharides have been described (US 6,005,099 and EP 0 729 473 B1; Hilgers *et al.*, 1986, *Int.Arch.Allergy.Immunol.*, 79(4):392-6; Hilgers *et al.*, 1987, Immunology, 60(1):141-6; and EP 0 549 074 B1).

A preferred form of 3 De-O-acylated monophosphoryl lipid A (3D-MPL) is in the
30 form of an emulsion having a small particle size less than 0.2µm in diameter, disclosed in International Patent Application No. WO 92/116556 (SmithKline Beecham Biologicals s.a.). See also WO 94/21292.

Aqueous formulations comprising monophosphoryl lipid A and a surfactant have been
35 described in WO98/43670A2.

Saponins are taught in: Lacaille-Dubois, M and Wagner H. (1996. A review of the biological and pharmacological activities of saponins. *Phytomedicine* vol 2 pp 363-386). Saponins are steroid or triterpene glycosides widely distributed in the plant and

marine animal kingdoms. Saponins are noted for forming colloidal solutions in water which foam on shaking, and for precipitating cholesterol. When saponins are near cell membranes they create pore-like structures in the membrane which cause the membrane to burst. Haemolysis of erythrocytes is an example of this phenomenon,
5 which is a property of certain, but not all, saponins.

Saponins are known as adjuvants in vaccines for systemic administration. The adjuvant and haemolytic activity of individual saponins has been extensively studied in the art (Lacaille-Dubois and Wagner, *supra*). For example, Quil A (derived from
10 the bark of the South American tree *Quillaja Saponaria* Molina), and fractions thereof, are described in US 5,057,540 and "Saponins as vaccine adjuvants", Kensil, C. R., *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; and EP 0 362 279 B1. Particulate structures, termed Immune Stimulating Complexes (ISCOMS), comprising fractions of Quil A are haemolytic and have been used in the manufacture of vaccines
15 (Morein, B., EP 0 109 942 B1; WO 96/11711; WO 96/33739). The haemolytic saponins QS21 and QS17 (HPLC purified fractions of Quil A) have been described as potent systemic adjuvants, and the method of their production is disclosed in US Patent No. 5,057,540 and EP 0 362 279 B1. Other saponins which have been used in systemic vaccination studies include those derived from other plant species such as
20 *Gypsophila* and *Saponaria* (Bomford *et al.*, *Vaccine*, 10(9):572-577, 1992).

QS21 is a Hplc purified non toxic fraction of a saponin from the bark of the South American tree *Quillaja Saponaria* Molina and its method of its production is disclosed (as QA21) in US patent No. 5,057,540.
25

Oil emulsion adjuvants have been known for many years, including work on Freund's complete and incomplete mineral oil emulsion adjuvants. Since that time much work has been performed to design stable and well tolerated alternatives to these potent, but reactogenic, adjuvant formulations.
30

Many single or multiphase emulsion systems have been described. Oil in water emulsion adjuvants *per se* have been suggested to be useful as adjuvant compositions (EP O 399 843B), also combinations of oil in water emulsions and other active agents have been described as adjuvants for vaccines (WO 95/17210). Other oil emulsion
35 adjuvants have been described, such as water in oil emulsions (US 5,422,109; EP 0 480 982 B2) and water in oil in water emulsions (US 5,424,067; EP 0 480 981 B).

In order for any oil in water composition to be suitable for human administration, the oil phase of the emulsion system preferably comprises a metabolisable oil. The

- meaning of the term metabolisable oil is well known in the art. Metabolisable can be defined as "being capable of being transformed by metabolism" (Dorland's Illustrated Medical Dictionary, W.B. Sanders Company, 25th edition (1974)). The oil may be any vegetable oil, fish oil, animal oil or synthetic oil, which is not toxic to the
- 5 recipient and is capable of being transformed by metabolism. Nuts (such as peanut oil), seeds, and grains are common sources of vegetable oils. Synthetic oils are also part of this invention and can include commercially available oils such as NEOBEE® and others. Squalene (2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene) is an unsaturated oil which is found in large quantities in shark-liver oil, and in lower
- 10 quantities in olive oil, wheat germ oil, rice bran oil, and yeast, and is a particularly preferred oil for use in this invention. Squalene is a metabolisable oil virtue of the fact that it is an intermediate in the biosynthesis of cholesterol (Merck index, 10th Edition, entry no.8619).
- 15 The oil in water emulsions which form part of the present invention when formulated with 3 D-MPL and QS21 are preferential stimulators of IgG2a production and TH1 cell response. This is advantageous, because of the known implication of TH₁ response in cell mediated response. Indeed in mice induction of IgG2a is correlated with such an immune response.
- 20 The observation that it is possible to induce strong cytolytic T lymphocyte responses is significant as these responses, in certain animal models have been shown to induce protection against disease.
- 25 The present inventors have shown that the combination of the adjuvants QS21 and 3D-MPL together with an oil in water emulsion with an antigen results in a powerful induction of CS protein specific CTL in the spleen. QS21 also enhances induction of CTL on its own, while 3D-MPL does not.
- 30 Induction of CTL is easily seen when the target antigen is synthesised intracellularly (e.g. in infections by viruses, intracellular bacteria, or in tumours), because peptides generated by proteolytic breakdown of the antigen can enter the appropriate processing pathway, leading to presentation in association with class I molecules on the cell membrane. However, in general, pre-formed soluble antigen does not reach
- 35 this processing and presentation pathway, and does not elicit class I restricted CTL. Therefore conventional non-living vaccines, while eliciting antibody and T helper responses, do not generally induce CTL mediated Immunity. The combination of the two adjuvants QS21 and 3D-MPL together with an oil in water emulsion can

overcome this serious limitation of vaccines based on recombinant proteins, and induce a wider spectrum of immune responses.

- CTL specific for CS protein have been shown to protect from malaria in mouse model systems (Romero et al. Nature 341:323 (1989)). In human trials where volunteers were immunised using irradiated sporozoites of *P. falciparum*, and shown to be protected against subsequent malaria challenge, induction of CTL specific for CS epitopes was demonstrated (Malik et al. Proc. Natl. Acad. Sci. USA 88:3300 (1991)).
- 10 The ability to induce CTL specific for an antigen administered as a recombinant molecule is relevant to malaria vaccine development, since the use of irradiated sporozoites would be impractical, on the grounds of production and the nature of the immune response.
- 15 In certain systems, the combination of 3D-MPL and QS21 together with an oil in water emulsion have been able to synergistically enhance interferon γ production.

IFN- γ secretion is associated with protective responses against intracellular pathogens, including parasites, bacteria and viruses. Activation of macrophages by IFN- γ enhances intracellular killing of microbes and increases expression of Fc receptors. Direct cytotoxicity may also occur, especially in synergism with lymphotoxin (another product of TH1 cells). IFN- γ is also both an inducer and a product of NK cells, which are major innate effectors of protection. TH1 type responses, either through IFN- γ or other mechanisms, provide preferential help for IgG2a immunoglobulin isotypes.

Particularly preferred adjuvants which may be used in the invention described herein are combinations of 3D-MPL and QS21 (EP 0 671 948 B1), oil in water emulsions comprising 3D-MPL and QS21 (WO 95/17210, PCT/EP98/05714), 3D-MPL formulated with other carriers (EP 0 689 454 B1), or QS21 formulated in cholesterol containing liposomes (WO 96/33739), or immunostimulatory oligonucleotides (WO 96/02555).

RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of *P. falciparum* linked via four amino acids of the preS₂ portion of Hepatitis B surface antigen to the surface (S) antigen of hepatitis B virus (HBV). The structure of RTS and the molecules from which it is derived is disclosed in International Patent Application Publication Number WO 93/10152.

When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS,S.

Liver Stage Antigens are described in Malaria, Parasite Biology, Pathogenesis and
5 Protection (1998 ASM Press, Washington D.C., edited by Irwin W. Sherman),
especially Chapter 34 (P. Druilhe et al.).

A 26-amino acid synthetic peptide based on Plasmodium falciparum liver stage
antigen 3 (LSA-3) is described in Eur J. Immunol., 1997, 27, 1242-1253 (L.
10 BenMohamed et al).

The immunogenicity of 12 synthetic peptides derived from four new Plasmodium
falciparum molecules expressed at pre-erythrocytic stages of the human malaria
parasite was reported in Vaccine 18 (2000), pages 2843-2855 (L BenMohamed et al).
15 In these studies the adjuvant Montanide ISA-51 (SEPPIC, Quai D'Orsay, France) was
used. There is no report, however, of such peptides being combined with other
adjuvants. The present invention is based on the surprising discovery that a Th-1
inducing adjuvant especially an oil in water emulsion which preferably comprises
tocopherol, as such or in combination with QS21 and/or 3 D-MPL (or related
20 molecules), enhances immune responses to a defined malaria antigen. Such
enhancement available affords better immunological responses than hitherto before.

According to the present invention there is provided a vaccine composition
comprising a Th1-inducing adjuvant in combination with a protecting Liver Stage
25 Antigen or immunological fragment thereof of a human malaria parasite with the
proviso that when the immunological fragment is an immunological fragment of LSA-
3, the Th1-inducing adjuvant is not Montanide.

In a preferred aspect of the invention the Th1-inducing adjuvant comprises QS21, De-
30 O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein
the oil in water emulsion has the following composition: a metabolisable oil, such a
squalene, alpha tocopherol and tween 80.

Normally the vaccine composition according to any aspect of the invention invokes a
35 T cell response in a mammal to the antigen or antigenic composition and is preferably
capable of stimulating interferon γ production. The oil in water emulsion used in the
present invention may be utilised on its own or with other adjuvants or immuno-
stimulants and therefore an important embodiment of the invention is an oil in water

formulation comprising squalene or another metabolisable oil, alpha tocopherol, and tween 80. The oil in water emulsion may also contain span 85 and/or Lecithin.

The combination of the two adjuvants QS21 and 3D-MPL together with an oil in water emulsion is particularly preferred. This is known and referred to herein as SBAS2, or alternatively simply as AS2 or AS02.

The ratio of QS21 : 3D-MPL will typically be in the order of 1 : 10 to 10 : 1; preferably 1 : 5 to 5 : 1 and often substantially 1 : 1. The preferred range for optimal synergy is 2.5:1 to 1:1 3D MPL: QS21. Typically for human administration QS21 and 3D MPL will be present in a vaccine in the range 1 µg - 100 µg, preferably 10 µg - 50 µg per dose. Typically the oil in water will comprise from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% tween 80. Preferably the ratio of squalene: alpha tocopherol is equal or less than 1 as this provides a more stable emulsion. Span 85 may also be present at a level of 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain a stabiliser.

In an alternative preferred embodiment, the vaccine of the invention may advantageously comprise a vesicular adjuvant formulation comprising cholesterol, a saponin, and optionally an LPS derivative. In this regard the preferred adjuvant formulation comprises a unilamellar vesicle comprising cholesterol, having a lipid bilayer preferably comprising dioleoyl phosphatidylcholine, wherein the saponin and optionally the LPS derivative are associated with, or embedded within, the lipid bilayer. Preferably the vesicular adjuvant comprises both the saponin and the LPS derivative. More preferably, these adjuvant formulations comprise QS21 as the saponin, and 3D-MPL as the LPS derivative, wherein the ratio of QS21:cholesterol is from 1:1 to 1:100 weight/weight, and most preferably 1:5 weight/weight. Such adjuvant formulations are described in WO 96/33739 and EP 0 822 831 B, the disclosures of which are incorporated herein by reference. For example a suitable formulation may contain 0.25 mg cholesterol, 1 mg dioleoyl phosphatidylcholine, 5 µg 3D-MPL, and 50 µg QS21 and consist of small lamellar vesicles wherein the saponin (QS21) and the LPS-derivative (3D-MPL) are in the membranes of the vesicles.

It will be appreciated that variants or derivatives of QS21 and 3-DMPL as described above may also be used without departing from the spirit of the invention.

The bacterial lipopolysaccharide derived adjuvants to be formulated in the adjuvant combinations of the present invention may be purified and processed from bacterial sources, or alternatively they may be synthetic. Accordingly, the LPS derivatives that may be used in the present invention are those immunostimulants that are similar in structure to that of LPS or MPL or 3D-MPL. In another aspect of the present invention the LPS derivatives may be an acylated monosaccharide, which is a sub-portion of MPL. In a preferred aspect the 3-DMPL is small particle 3-DMPL as described in WO 92/116556.

The oil emulsion adjuvants for use in the present invention may be natural or synthetic, and may be mineral or organic. Examples of mineral and organic oils will be readily apparent to the man skilled in the art based on the description hereinabove.

Particularly preferred oil emulsions are oil in water emulsions, and in particular squalene in water emulsions.

In addition, the most preferred oil emulsion adjuvants of the present invention comprise an antioxidant, which is preferably the oil α -tocopherol (vitamin E, EP 0 382 271 B1).

WO 95/17210 discloses emulsion adjuvants based on squalene, α -tocopherol, and TWEEN 80, optionally formulated with the immunostimulants QS21 and/or 3D-MPL.

The size of the oil droplets found within the stable oil in water emulsion are preferably less than 1 micron, may be in the range of substantially 30-600nm, preferably substantially around 30-500nm in diameter, and most preferably substantially 150-500nm in diameter, and in particular about 150 nm in diameter as measured by photon correlation spectroscopy. In this regard, 80% of the oil droplets by number should be within the preferred ranges, more preferably more than 90% and most preferably more than 95% of the oil droplets by number are within the defined size ranges. The amounts of the components present in the oil emulsions of the present invention are conventionally in the range of from 2 to 10% oil, such as squalene; and when present, from 2 to 10% alpha tocopherol; and from 0.3 to 3% surfactant, such as polyoxyethylene sorbitan monooleate. Preferably the ratio of oil: alpha tocopherol is equal or less than 1 as this provides a more stable emulsion. Span 85 may also be present at a level of about 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain a stabiliser. Preferably the oil emulsion contains a surfactant such as polyoxyethylene sorbitan monooleate (TWEEN80™), but it will be clear to the man skilled in the art that other surfactants

may be used, preferred examples of which are the SPAN series (especially SPAN85) and or lecithin.

5 The method of producing oil in water emulsions is well known to the man skilled in the art. Commonly, the method comprises the mixing the oil phase with a surfactant such as a PBS/TWEEN80™ solution, followed by homogenisation using a homogenizer, it would be clear to a man skilled in the art that a method comprising passing the mixture twice through a syringe needle would be suitable for homogenising small volumes of liquid. Equally, the emulsification process in
10 microfluidiser (M110S microfluidics machine, maximum of 50 passes, for a period of 2 minutes at maximum pressure input of 6 bar (output pressure of about 850 bar)) could be adapted by the man skilled in the art to produce smaller or larger volumes of emulsion. This adaptation could be achieved by routine experimentation comprising the measurement of the resultant emulsion until a preparation was achieved with oil
15 droplets of the required diameter.

In a preferred aspect of the invention the human malaria parasite is *Plasmodium falciparum*.

20 In a particular aspect of the invention the said protecting Liver Stage Antigen is the Liver Stage Antigen 3 (LSA-3) or immunological fragment thereof.

However other Liver Stage Antigens may also be used, for example LSA-1 and LSA-2 as described in *Malaria, Parasite Biology, Pathogenesis and Protection* (1998 ASM Press, Washington D.C., edited by Irwin W. Sherman), especially Chapter 34 (P. Druilhe et al.).

By immunological fragment is meant herein a molecule which has a related or similar sequence to the reference antigen in terms of % homology and which can induce a
30 similar immune response, cellular or humoral, in vivo.

The LSA-3 antigen and polypeptide molecules containing at least 10 consecutive amino acids of the amino acid sequence representing LSA-3 are described in WO 96/41877. LSA-3 for use in the present invention may suitably be prepared as
35 described in the examples section of the present specification. Reference may also be made to C Marchand and P Druilhe, *Bulletin of the World Health Organisation*, Volume 68 (Suppl.) 158-164 (1990) and US Patent Number 6,100,067.

In a further aspect there is provided a vaccine composition according to the invention comprising in addition at least one other protecting antigen or an immunological fragment thereof, of a malaria parasite, in particular LSA-3.

5 In particular, the other malaria antigen may be selected from the following group:

- a) a hybrid protein comprising substantially all the C-terminal portion of the CS protein, four or more tandem repeats of the immunodominant region, and the surface antigen from hepatitis B virus (HBsAg), in particular RTS,S, or an
10 immunogenic derivative including fragments thereof;
- b) the TRAP protein of the T9/96 isolate of *Plasmodium falciparum* and proteins having at least 80% homology thereto and immunogenic derivatives including fragments thereof (see European Patent Application No 91903249.0);
- c) the MSP-1 of *Plasmodium falciparum* or *Plasmodium vivax* and proteins
15 having at least 80% homology thereto and immunogenic derivatives including fragments thereof ; and
- d) the MSP-3 of *Plasmodium falciparum* or *Plasmodium vivax* and proteins having at least 70% homology with the C-terminal region thereof, and immunogenic derivatives including fragments thereof.

20

MSP-1 of *P.falciparum* or *P.vivax* is described in US Patent No. 4,837,016.

Immunogenic derivatives include fragments thereof such as the C-terminal 42 KDa antigen (p42).

25 The MSP-3 antigen is described in US Patent Number 6,017,538.

Homology in sequence analysis may be established by the use of Blast 2.0 and Fasta default settings of the algorithms used by these programs. The comparison of LSA-3 sequences in various isolates or stocks can be done using a calculation manual.

30

By C-terminal region of MSP-3 is meant a 185 amino acid region from positions 193 to 381. It contains a leucine zipper on its extremity (C-terminus part) and is rich in acidic amino acids. The three-dimensional structure is coil-coiled. The clone DG 210 (amino acids 193-257) corresponds to a globular region of high complexity and is
35 followed by the coil-coiled region.

In a further aspect of the present invention there is provided a vaccine as herein described for use in medicine.

In yet a further aspect the invention provides a process for making a vaccine composition according to any aspect of the present invention by mixing the required components using standard techniques. Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978.

In one aspect the process comprises admixing QS21, 3D-MPL and the oil in water emulsion with a protecting Liver Stage Antigen of a human malaria parasite as hereinabove defined, optionally with an additional malaria antigen.

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000ug of protein, preferably 2-100 ug, most preferably 4-40 ug. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced.

The formulations of the present invention maybe used for both prophylactic and therapeutic purposes.

Accordingly in one aspect, the invention provides a method of treatment comprising administering an effective amount of a vaccine of the present invention to a patient.

The following examples illustrate the invention.

Examples

Example 1

Two adjuvant formulations were made each comprising the following oil in water emulsion component.

SB26: 5% squalene 5% tocopherol 0.4% tween 80; the particle size was 500 nm size
SB62: 5% Squalene 5% tocopherol 2.0% tween 80; the particle size was 180 nm

1(a) Preparation of emulsion SB62 (2 fold concentrate)

Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100 ml two fold concentrate emulsion 5g of DL alpha tocopherol and 5ml of squalene are vortexed to mix thoroughly. 90ml of PBS/Tween solution is added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

1(b) Preparation of emulsion SB26

This emulsion was prepared in an analogous manner utilising 0.4% tween 80.

To the emulsion of 1 a) or b) an appropriate amount of LSA-3 (for example 2µg to 100µg) may be added and mixed. This may be combined with, for example, 50µg/ml of 3D-MPL and 20µg/ml of QS21 (or related molecules) to give the final formulation.

Example 2

Protection against *Plasmodium falciparum* malaria in chimpanzees by immunisation with a conserved pre-erythrocytic antigen, LSA-3

The basis of the strong immunological protection induced in humans by vaccination with radiation-attenuated pre-erythrocytic malaria parasites is poorly understood. However it is now suspected that the transformation of the irradiated sporozoites into live but developmentally arrested intra-hepatic liver trophozoites is required to induce protection⁹. This occurs at low (15-20 krad) but not at high (23-30 krad) irradiation doses^{9,10}. We reasoned that the differential response of hosts immunised with such irradiated sporozoites could provide a screen for molecules relevant to protection. We proceeded to screen 120 phage lambda clones previously identified as expressing *P. falciparum* polypeptides that are expressed during pre-erythrocytic stage parasite development^{6,7} and which derive from ca. 20 distinct genes^{6,7,11,12}. A clone corresponding to each of these putative genes was screened using eight sera from human volunteers (4/6 protected) and from chimpanzees (1/2 protected) immunised with sporozoites irradiated at low or high doses. A single clone (DG729) reacted only with sera from protected humans and chimpanzees. This differential reactivity was further confirmed with a peptide derived from this fragment (Table I). This led us to select this clone for further investigation.

DG729 was used to probe a *P. falciparum* (K1) genomic library. One clone was found to contain the whole gene corresponding to DG729, and which was named Liver Stage Antigen-3 (LSA-3). Full description of the sequence, expression, location and conservation of the *lsa-3* gene is provided in the Supplementary Information (S.I.) and is summarised below and in Figures 1-3. Briefly we identified a single-copy gene which comprises a mini-exon 1, a mini-intron, and a large exon 2 (Fig. 1a), a structure similar to that of other surface antigens of *P. falciparum*¹³. It was recently confirmed that *lsa-3* is located on chromosome 2¹⁴, where the gene was annotated as « RESA-H3 » gene (Acc. Number AE001424). LSA-3, with a predicted molecular weight of 200 kDa (in K1), is made up of large non-repeated sequences flanking three glutamic acid-rich repeated regions, a feature that extends the known *P. falciparum* Glu-rich antigen network¹⁵ to include a pre-erythrocytic component. The location of the original fragment (DG729) and of the peptides corresponding to the repeat region R2 and to the non-repetitive regions NR-A and NR-B are shown in Fig. 1b. Naturally- or artificially- induced antibodies against the non-repeated peptides and the recombinant protein GST-PC were not cross-reactive with the repeated Glu-rich regions, and were used for further studies.

Pre-erythrocytic expression of LSA-3 (see Fig. 2-3 and see S.I.) was confirmed a) by RT-PCR (primers i1 and i2) of total RNA and Western blotting of protein extracts, isolated in both cases from sporozoites, and b) by immunofluorescence antibody test (IFAT) on infected liver sections and dry or wet sporozoite preparations, using antibodies to a non-crossreactive portion of the protein. In the five and six day-old liver schizonts, LSA-3 was located in the parasitophorous vacuole and at the periphery of maturing hepatic merozoites. This location is consistent with the molecular structure of this protein, which contains two hydrophobic regions (Fig. 1a). In our hands, mRNA from *lsa-3* could not be detected in Northern blotted RNA from erythrocytic stages. Western blottings and IFAT of infected red blood cells were also consistently negative with non cross-reactive antibodies. Reactivity was however obtained when antibodies to the Glu-rich repeat region were used. This might explain in part the detection of a putatively homologous antigen (D260) previously described in intra-erythrocytic parasites, and which was identified solely using antibodies which cross-react extensively with Glu-rich epitopes¹⁶.

Polymorphism of many malaria vaccine candidate molecules is of recognised concern, we therefore investigated naturally occurring sequence variation in LSA-3 (see S.I.). The gene was consistently detected by PCR amplification of the NR-A region (primers S1 and S2) in a total of 111 *P. falciparum* isolates, strains or clones of various geographical origin. Using LSA-3 specific antibodies in IFAT assays, the expression of LSA-3 was also detected in liver schizonts of two distinct strains and in all the sporozoites from 30 wild isolates which developed in mosquitoes fed *in vitro*

on Thai gametocytes. The repeat regions R1 and R3 are highly conserved, but variation in the number and order of the repeat units of R2 was found to occur amongst different parasite lines. This did not however affect the predicted conserved ?-helical organisation, a secondary structure considered to be important in defining major B-cell epitopes since antibodies which recognise R2 did indeed react positively by IFAT with all the parasites tested. The non-repeated portions of exon 2, where numerous Th and CTL epitopes are found¹⁷⁻¹⁹, displayed a remarkable degree of amino acid (aa) sequence conservation between different parasites (>95.5% homology). The sequence of NR2 peptide was fully conserved amongst K1 and T9/96 parasites, the source of the immunising proteins, the NF54 parasites used for sporozoite challenges, and 27 *P. falciparum* samples of various geographical origin¹⁷. An HLA-B53 restricted epitope identified in the NR-B region of LSA-3 (present in GST-PC recombinant protein) was also found to be free of variation in clone 3D7 and in 18 Gambian isolates¹⁹. This conservation of immunologically important epitopes contrasts with substantial polymorphism in current pre-erythrocytic vaccine candidates.

We selected the chimpanzee to investigate the protective capacity of LSA-3 immunisation for the following reasons. The chimpanzee is the only non-human primate fully susceptible to complete intra-hepatic development of *P. falciparum*, with a comparable rate of sporozoite transformation to liver forms to that seen in humans⁹. The chimpanzee is also the most closely related animal to humans (98.4 % homology at the DNA level⁸), and one in which detailed investigations of immune responses can be performed and legitimately compared with those of humans^{17,18}. The fact that parasitological and immunological events can be directly examined in the liver biopsies, a possibility excluded for infected humans, is clearly of considerable significance. A number of preliminary stringent tests were conducted in control animals in order to validate the suitability of this model for vaccine evaluation. Since cost and ethical considerations preclude the use of large number of animals, high reproducibility of the infection in this model system is critical. In a preliminary experiment (Group I, Table II), we confirmed that in the chimpanzee protection by immunisation with irradiated sporozoite is radiation dose-dependent, and we validated the detection of the infected red blood cells as an assay of protection. The results allowed us to define a number of important parameters: a) as in humans, chimpanzees develop a powerful protective response following immunisation with irradiated sporozoite, b) chimpanzees, like humans, remain broadly susceptible to at least five successive challenges, in contrast to lower primates or rodents which become refractory after the first challenge²⁰, and c) as a result of the high dose of inoculated sporozoites detection of erythrocytic parasites corresponded to the first

invasion of red cells by merozoites released from intra-hepatocytic schizonts. Positive blood smears were reproducibly obtained in non-protected chimpanzees on days six or seven. In the chimpanzee erythrocytic infections normally remain sub-clinical and self-limiting which was in fact observed despite the high dose challenges. These results have been recently confirmed in two further chimpanzees (Langermans J. *et al*, manuscript in preparation).

Having established the suitability of the chimpanzee, we proceeded to assay the protective value of LSA-3 immunisation by challenge with viable *P. falciparum* sporozoites. In preliminary experiments, two animals were immunised with a mixture of LSA-3 and LSA-1 recombinant proteins. Full protection against three challenges over several months was only seen in the animal which responded to LSA-3 (both responded to LSA-1). In liver biopsies performed on this animal on day five, only one liver schizont of unhealthy appearance and infiltrated by leukocytes could be detected in the 300 liver sections screened (Dirk, Fig. 3). By contrast 2500 and 750 hepatic schizonts of healthy appearance were observed in the two non-protected controls.

These results led us to focus further immunisation and challenge experiments on LSA-3 alone. Two groups of chimpanzees were used to evaluate lipopeptide and recombinant protein formulations (Table II, Groups II-III). In Group II, one animal (Gerda) was initially immunised solely with the NR2 lipopeptide of LSA-3, and boosted by recombinant LSA-3 molecules in Montanide ISA 51. Gerda was fully protected when challenged with 10^7 sporozoites, whereas the control receiving Montanide ISA 51 was not (Fig. 4a).

In Gerda boosting with the recombinant LSA-3 formulation was not found to induce any detectable increase in the strong B-cell, T-helper cell and CTL responses already evoked by the initial lipopeptide/peptide injections^{17,18}. We were therefore interested to see whether the simple and well-tolerated peptidic formulation alone could induce protection. Two chimpanzees, Mopia and Mgbado were immunised with LSA-3 lipopeptides/peptides alone (Table II, Group III). Protection against a first challenge with 2×10^4 sporozoites was obtained in both. The same group included an investigation of the effects of microbead presentation of recombinant proteins without adjuvant in one animal (Judy) which resulted in a one-day delay to patency (Fig. 4b). Following a subsequent high dose sporozoite challenge (5×10^6 sporozoites), both Mopia and Mgbado demonstrated a clear two-day delay to patency and a low transient parasitaemia, whilst no protection was found for Judy (Fig. 4c). The delay to patency suggests that the immune responses had caused a reduction exceeding 90% of intra-hepatocytic schizont load²¹ (Fig. 4).

In chimpanzees from groups IV and V, we investigated the efficacy of a less complex lipopeptide mixture alone, or of recombinants adjuvated by SBAS2, a novel adjuvant whose efficacy has been recently established in humans^{4,5}. Since

immunogenicity studies^{17,18} and analysis of previous chimpanzee data had indicated that peptide CT1 was poorly immunogenic and thus might not be critical, chimpanzee Patty was immunised by a mix of three instead of four peptides. This animal showed protection upon challenge. Among four animals receiving SBAS2 adjuvated LSA-3 proteins, two showed full, sterile protection against a medium dose challenge. One showed a delay in patency which may be indicative of partial protection, whereas neither the fourth nor the control receiving SBAS2 adjuvant alone were protected. One of the two fully protected chimpanzees was further challenged with a high dose three months later and still showed full protection.

We present here the first description of protective vaccination against human malaria in the chimpanzee. This model provided us with convincing evidence that LSA-3 of *P. falciparum* is a valuable candidate for effective vaccination against pre-erythrocytic stages. A total of nine animals were immunised using lipopeptides in saline or polypeptides in either Montanide or SBAS2 adjuvants. Full sterile protection was induced in six of these nine chimpanzees on first challenge. If the significant delay as compared to controls is taken in consideration, a protective effect induced by LSA-3 was shown in eight of nine animals. Out of the 14 challenges which were performed, complete protection was obtained in seven, and partial protection in an additional four challenges. All seven control animals employed in these studies showed a consistent pattern in the appearance and the course of the blood-stage parasitaemiae following each of the 12 challenges with viable parasites. Demonstration of this reproducibility in controls, in animals immunised by over-irradiated sporozoites, and in an additional 26 challenges performed in other experiments (not shown), is an essential point in the interpretation of our data.

It is encouraging that protection was induced against a heterologous challenge (NF54) in outbred animals immunised with LSA-3 molecules whose sequences were derived from K1 and T9/96 parasites. A variety of immunisation strategies were investigated in the course of this work. The data underpin the value of the SBAS2 adjuvant. The results with Gerda, Mopia, Mgbado and Patty are also particularly encouraging since they are based on simple peptide and lipopeptide formulations which are relatively easy to produce under GMP conditions²². In our animals no local or general reactions was detected following lipopeptide injections, an observation consistent with previous experience with similar formulations derived from SIV in macaques²³ and HbS²⁴ or HIV²² in humans. This bodes well for future clinical trials.

METHODS

Selection of clone DG729. Dot blot analysis of the β -galactosidase-fused recombinant proteins encoded by the pre-erythrocytic clones was performed on nitrocellulose as previously described⁷, using 1/100 diluted human and chimpanzee sera. ELISA was

performed in duplicate as previously described²⁵ on 1/100 diluted sera using coating solutions of 0.3, 3 and 10 µg/ml of NR1, NR2 and RE peptides respectively, in PBS.

LSA-3 cloning and characterisation. Detailed description of molecular methods, gene cloning, sequence data, protein characteristics and description of the

5 recombinant proteins and of the peptides are provided in the S.I. The primers used for PCR: S1 (nucl.161-184)/S2 (nucl.454-432) and for RT-PCR: i1 (nucl.695-722)/i2 (nucl.824-799), numbering refers to the *lsa-3* sequence of K1 (Accession Nber AJ007010). All mouse sera used for the Western blot (at dilution 1/100) presented in Fig. 2 were obtained following 3 subcutaneous injections of the immunogen (100 µg) 10 emulsified in SBAS2 adjuvant⁴. Long synthetic peptides GP5, GP6, GP8 and GP11 were synthesised as described in ref. 26 (see Fig. 1 for position).

Immunogens injected in chimpanzees. Sequences of the various immunogens evaluated here consisted of clone DG729 and inserts NN and PC, as well as peptides (pep.) NR1, NR2, RE and CT1; their location is shown in Fig. 1 and described in 15 more details in the S.I. Clone DG729, as well as inserts NN and PC were expressed as glutathione-S-transferase-fused recombinants and purified according to manufacturer recommendations (Invitrogen, The Netherlands). Recombinants GST-DG729, -NN and -PC were designed so as to cover 95% of the LSA-3 antigen and were used as a mixture mentioned as LSA-3 GST-rec. Peptides NR1, NR2 and CT1, were also 20 synthesised as palmitoyl-conjugated lipopeptides (lipopep.), as described in ref. 17. Combination of synthetic compounds (mentioned as (lipo)pep.) consisted in a mixture of NR1, NR2 and CT1 lipopeptides and of RE peptide. All peptides and lipopeptides were purified to >90% purity by reversed-phase chromatography, and the impurities consisted essentially of related peptides of shorter sequences¹⁷.

25 **Chimpanzee immunisations and challenges.** None of the chimpanzees included in this study had previously been exposed to malaria infections or malarial antigens. Recombinant and synthetic compounds were injected subcutaneously, at a dose of 100 µg for each peptide and/or lipopeptides, and/or 50 µg for each protein. Lipopeptides were always injected in PBS and, except when mentioned, peptides and recombinants 30 were emulsified in Montanide ISA51. Group I animals (Carl and Japie) were immunised by five intra-venous injections of 5×10^6 gamma-irradiated sporozoites at day 0 and weeks 8, 24, 44 and 65, and received three challenges at weeks 71, 97 and 123 (challenge doses are given in Table II). One year after the three challenges reported here, these chimpanzees were re-immunised once, and received one low and 35 one high dose challenges, which revealed the same pattern of protection (not shown, Langermans J. *et al.*, manuscript in preparation). In Group II, Gerda received NR2 lipopeptide at day 0 and weeks 3, 13 and 31 as described in ref. 17. She was then boosted with the mixture of LSA-3 GST-rec. at weeks 40, 45, 48 and 50. Control animal Lianne received Montanide ISA51. Challenges were performed at week 60.

Group III animals were immunised at day 0 and weeks 3 and 6. Mopia and Mgbado received LSA-3 (lipo)peptides whereas Judy was injected with LSA-3 GST-rec. adsorbed to latex microbeads. Challenges LD and HD were performed at weeks 21 and 29. In Group IV, Patty received LSA-3 (lipo)peptides, but without lipopeptide CT1, whereas Wendy and Willy were injected with LSA-3 GST-rec in SBAS2 adjuvant^{4,5}. Control animal Helen received SBAS2 adjuvant only. All animals were immunized at weeks 0, 4 and 8 and were challenged with 20,000 sporozoites at week 13. In Group V, Cindy and Marty were both immunised at weeks 0, 4, 8 and 26 with LSA-3 GST-rec in SBAS2 adjuvant (as in Group IV) and negative control animal Fauzi received over-irradiated sporozoites similarly to Japie (Group I) at weeks 5, 8, 11 and 26. Challenges LD and HD were performed at weeks 33 and 46 in all three animals.

NF54 sporozoites were obtained from dissected salivary glands of infected *Anopheles gambiae* as previously described²⁷. Sporozoites were pooled, resuspended in PBS and injected intravenously. All animals in each group were challenged with the same pool of sporozoites. For cost reasons, extensive evaluation of the Minimal Infective Dose has not been undertaken, however challenge with 5×10^3 sporozoites, the lowest dose used to date, has proven infective in four other animals (Thomas, A.W., unpublished data).

Determination of the protective status. For Groups I, II, IV and V, animals blood was taken on days five to nine, and evaluated by thick and thin film Giemsa-stained preparations, and confirmed in all cases by *in vitro* culture (not shown), as described in ref. 21. For Group III chimpanzees blood taken every day from day five up to day 18, then every other day up to day 30, was used to prepare thin and thick smears which were Giemsa-stained and examined by two separate microscopists. A chimpanzee was considered a) totally protected when no parasites could be detected in the circulation blood, by direct microscopical observation and by long term culture, or b) partially protected when time to patency was delayed by one or more days as compared to that observed in control animals. In mice, these delays correspond to a protection of 80% (24h) or 96% (48h) against sporozoite challenges. In humans, a 12 hour delay was calculated to correspond to a 92% reduction of liver forms following sporozoite challenges²¹. In a limited number of animals a liver biopsy was performed under anaesthesia by a veterinary doctor on day five following a high dose challenge. Material was fixed and 4 μ m sections were made and stained by Giemsa-collophonium²⁸ before complete microscopic enumeration of the liver forms in 300 sections (average area 0.8 cm²). All animals were curatively treated with chloroquine immediately after the period of observation, and irrespective of their protective status.

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35 Example 3

The following experiments take advantage of the long peptide strategy (LSP) developed by GianPietro Corradin in Lausanne, which allow one to establish proof of

concept at clinical level by producing in short time and at low cost Long Synthetic Peptides. These are in fact short proteins which can be employed in clinical trials. A series of 17 overlapping Long Synthetic Peptides was synthesised covering the full length of the LSA-3 molecule.

- 5 These peptides were used in antigenicity studies at T-cell and B-cell level in exposed individuals in the field in Senegal to monitor antibody and lymphoproliferative responses to each of them in exposed populations. They were used also to immunise mice using AS2 adjuvant.

Both studies demonstrated a very strong antigenicity of most of the peptides which, each, defined at least one B-cell and one T-cell epitope and immunogenicity studies in mice indicated that most peptides studied were also strongly immunogenic to laboratory mice (summarised in : Perlaza et al. European Journal of Immunology, 10 2001 Jul;31,7,2200-9)

Challenge experiments with the cross-reactive *Plasmodium* of rodents, *Plasmodium* 15 *yoelii*, indicated in particular that a peptide called GP1 could induce protection against virulent *P.yoelii* sporozoite challenge. For further studies in humans, to chose the immunizingg peptides we relied on initial results obtained with the recombinant denominated DG729 which overlaps part of the non-repetitive N-terminal region of the molecule and the beginning of the repeat region.

20 Two types of formulations were investigated :

a) A very long LSP of ca 160 aminoacids, covering the end of the Non-repeated region, including the short peptides NR1 and NR2 investigated formerly and the beginning of the repeat region.

b) A mix of 2 peptides, one covering only the non-repeat region, called GP1 and 25 another, called GP14, located in the beginning of the repeat region.

For practical reasons, it was found that it would be difficult to produce in sufficiently pure form the very long species mentioned above in a), and that for GMP production it would be safer to rely on a mix of the two peptides mentioned in b), namely GP1 and GP14.

30 Therefore, pre-clinical studies were performed in South-american primates, *Aotus trivirgatus griseimembra*, by Blanca-Liliana Perlaza in the collaborative laboratory of Socrates Herrera in Cali, Colombia.

7 animals were enrolled in this study as follows:

- Group 1 : 2 animals receiving 3 injections of LSA-3 GP1 LSP at a dose of 50 35 mg per injection per animal, adjuvated by AS2 in a total volume of 500 ml per injection.
- Group 2 : 2 animals receiving 3 injections of a mixture of peptides LSA-3 GP1 + LSA-3 GP14, at a dose of 50 mg of each peptide per injection per animal, adjuvated by AS2 in a volume of 500 ml per injection.

- Group 3 : 2 animals receiving 3 injections of PBS with adjuvant AS2 in a volume of 500 µl per injection, plus 1 non-immunised control.

One month after the last immunisation, which were well tolerated and did not induce any major local or general reactions, blood samples were taken to analyse

- 5 immunogenicity : results are shown in the corresponding graphs and demonstrated strong antibody production, lymphoproliferative responses and Interferon-γ production, both in culture supernatant of lymphocytes and by Elispot technique. Animals were challenged by intra-venous inoculation of 100 000 sporozoites of the Santa Lucia strain of *Plasmodium falciparum* 3 months after the last immunisation.
- 10 Blood samples taken over a period of 60 days after challenge may be analysed by 3 different techniques, namely microscopy of coloured blood, Polymerase Chain Reaction and LDH assay.

- The study of the degree of protection achieved by the LDH assay has been completed. This method relies on the detection of the parasite by a double-site ELISA capture
- 15 assay which has been recently described (Druilhe et al., American Journal, 64 (5, 6) 2001, 233-241) and which was shown to be at least 10 times more sensitive than microscopy. The results obtained are presented in the figures. They essentially show that the 3 control animals became blood-stage positive, i.e. yielded positive parasite-specific LDH detection during the follow-up, whereas the 2 immunised groups
- 20 remained consistently negative by this technique over the 60 days of follow-up.

- The results support the protection induced by immunisation by the GP1 LSP or the GP1 + GP14 LSPs adjuvanted by AS2. These results are in agreement with previous data obtained using the recombinant DG729 alone which covers the same region of
- 25 the antigen, as well as immunisation performed by a mix of lipopeptides covering the same region, as well as those obtained by a mix of 3 recombinants (729, NN, PC) adjuvanted by AS2 (Daubersies et al., Nature Medicine, Nov. 2000, 6, 11, 1258-1263). The sequence of the 2 peptides employed is shown below.

30 *GP1*

L A S E E V K E K I L D L L E E G N T L T E S V D D N K N L E E A E D I
K E N I L L S N I E E P K E N I I D N L L N N I G Q N S E K Q E S V S E N
V Q V S D E L F N E L L N S V D V N G E V K E N I L E E S Q V N D D I F
35 N S L V K S V Q Q E Q Q H N

GP 14

ESVAENVEES VAENVEEIVAPTVEEIVAPTVEEIVAPSVV ESVAPSVEES
40 VEENVEESVA ENVEESVAEN VEESVAENVEESVAENVEEI VAPTVE

Code or Name	Spz. irrad. dose	IFAT titers on spz.	status	NR2 peptide (aa 198- 223)
V4	23.6	4,096	not	0.5
V5	23.6	32,000	protected	0.5
<i>Japie</i>	30	3,200	2 day delay not	0.7
V6	20.8	5,120	protected Protected	3.8
V7	20.8	41,960	Protected	2.6
V8	20.8	40,960	Protected	4.8
WR4	15	3,200	Protected	3.4
<i>Carl</i>	18	6,400	Protected	2.3

5 Spz.: sporozoite; irrad.: irradiation

Table I (above): Differential reactivity of sera from protected or non-protected humans or chimpanzees with peptide NR2. IgG-specific antibodies against peptide NR2 were measured by ELISA in sera from human volunteers (codes) and chimpanzees (names in *italic*) immunised with sporozoites irradiated at low or high dose (in krad). Codes, immunisation schemes, sporozoite IFAT titres and protective status determination for human volunteers V4-V8 and WR4 are detailed in ref. 1 and 2, respectively. Chimpanzees Carl and Japie were immunised and challenged as described in the text and the Methods (Group I). ELISA titres are expressed in arbitrary units representing the ratio of the mean ODs from test sera to the mean OD plus three standard deviations from 10 controls studied in parallel in the same plate. Results are taken as positive for ratios above one (in bold). Similar experiments performed with peptides NR1 and RE (see Fig. 1) yielded negative results with these sera (not shown).

ANIMAL GROUPS			PROTECTION	
Chimp.	Immunisation protocols ^a	Immunisation and challenge dates (weeks)	LD 2x10 ⁴	HD 10 ⁷
Carl	Group I ^b 18 krad-irradiated sporozoites	97 123 [0-8-24-44-65]	+	+
Japie	30 krad-irradiated sporozoites	60	-	-
Marcel	unimmunised control	[0-5-15-31] [40-45-48-50]	-	-
Theo	unimmunised control		-	-
Gerda	Group II [lipopep. NR2] then [GST-rec. in ISA51]	21 29 ^c [0-3-6]	nd	+
Lianne	control ISA 51		nd	-
Mopia	Group III [(lipo)pep.]	13 [0-4-8]	+	d2
Mgbado	[(lipo)pep.]	33 46	+	d2
Judy	[GST-rec. / microbeads]	[0-4-8-26]	d1	-
Ondele	control GST / microbeads	[5-8-11-26] ^f	-	-
Makata	unimmunised control		-	-
Patty	Group IV [(lipo)pep.] ^d		+	nd
Wendy	[GST-rec. in SBAS2]		+	nd
Willy	[GST-rec. in SBAS2]		-	nd
Helen	control SBAS2		-	nd
Cindy	Group V [GST-rec. in SBAS2]		+	+
Marty	[GST-rec. in SBAS2]		d1	-
Fauzi	30 krad-irradiated sporozoites		-	-

Chimp.: chimpanzee name; HD/LD: high/low dose sporozoite challenges; d1/d2: one/two-day delay to patency; nd: not done.

a) details and abbreviations are given in the Methods.

b) Group I chimpanzees received three additional challenges (2 LD and 1 HD) which led each time to similar results, i.e. a reproducible protection only in Carl (data not shown).

c) HD challenge was performed with 5×10^6 sporozoites.

d) same mixture as in Group III but without peptide CT1.

e) performed in Cindy and Marty.

f) performed in Fauzi.

Table II (above): Immunisation and challenge experiments in the chimpanzees.

Challenges were performed with either 2×10^4 (low dose) or 10^7 (high dose) NF54 *P. falciparum* sporozoites ("Protection" column). Immunisation schedules (in brackets under the bar) and of challenges (indicated by arrows above the bar) are expressed in weeks from first immunisation. Complete protection is indicated with (+); a delay to patency (in days) as compared to controls and non-protected animals is indicated by d1 or d2 (determination of the protective status is detailed in the Methods).

LEGENDS FOR FIGURES

Figure 1: Schematic representation of the LSA-3 gene, recombinant proteins and peptides. a) 6.2 Kb *Eco* RI-insert isolated from K1 parasite genomic DNA library that hybridised with DG729. The 5.53 Kb gene comprises a 198 bp exon 1, a 168 bp intron (i) and a 5.16 Kb exon 2. Regions NR-A, -B and -C correspond to non-repeated sequences whereas R1 to R3 designate the three repeat blocks. The two hydrophobic regions potentially corresponding to the NH₂-terminal signal peptide and the anchor region are indicated by HR1 and HR2 respectively. b) Location of the sequences encoding for LSA-3 in the recombinant fusion proteins (first line) and the synthetic peptides (strokes) used in this study (see Supplementary Information for aa numbering). For the immunisations, CT1 and NR2 were also used as palmitoyl-conjugated lipopeptides¹⁷ (indicated by *).

Figure 2: LSA-3 expression in *P. falciparum* sporozoites. Western blot analysis was performed on protein extracts from NF54 sporozoites and control uninfected mosquito salivary glands using mouse antisera directed against: C) control GST, 1) GST-PC, 2) peptides GP5, GP6, GP8 or GP11, 3) GST-729 (see Fig. 1, Methods and S.I.). LSA-3 is visualised as a 175 kDa protein (*), in agreement with the theoretical molecular weight of LSA-3 in this parasite strain.

Figure 3: Immunostaining of *P. falciparum* pre-erythrocytic stages with anti-LSA-3 antibodies. a) sporozoites stained by IFAT with human antibodies affinity purified on recombinant β gal-DG729. b) staining by IFAT of day six post-challenge liver stages²⁹ from a chimpanzee, using the antibodies induced by lipopeptide NR2 injection¹⁷ in chimpanzee Gerda (see S.I. for additional pictures). c) The single residual liver schizont detected in a chimpanzee Dirk (day five post-challenge) appeared infiltrated by lymphomononuclear cells, as compared in d) to one of the numerous healthy schizonts observed in the control chimpanzee Peer (total of ca 2500 schizonts/300 liver sections, Giemsa-collophonium staining²⁸) (see text). Bars correspond to 5 μ m in panel a) and 20 μ m in panels b) to d).

Figure 4: Blood parasitaemia courses in Groups II and III. a) chimpanzees from Group II and b-c) animals in Group III, following high dose (HD) or low dose (LD) challenges with NF54 sporozoites. Names of totally or partially protected animals are in bold. Hatched patterns correspond to control chimpanzees. Parasitaemia scales are different for each challenge, as expected from challenges with different numbers of sporozoites. Note that the day of patency in control and non-protected animals was the same for a given challenge inoculum within each group (in the above and in other groups not shown here).

Figure 5 : antibody titers following immunisation with GP1 and GP14

The figure shows the results from ELISA assays of Aotus M73 and D114 immunised by LSA-3 GP1 + adjuvant AS2 against the immunising peptide GP1 or the recombinant DG729. In both cases, the titers are high as the result is significant for values higher than an ELISA ratio of 1. The second half of the figures show the results obtained in Aotus M88 and M91 immunised by GP1 + GP14 adjuvated by AS2, against peptide GP1 and GP14 or the recombinant 729 or NN covering the repeat region, or a control recombinant (GST). Here again, the responses are high against both immunising peptides.

Figure 6: Proliferative responses to GP1 and GP14

The figure shows the proliferative responses in M88 and M91 and M223 (a third animal, included in fact in group 2, but not challenged) of monkeys immunised by a mix of GP1 + GP14 with AS2 adjuvant. Significant proliferative responses were obtained to the immunising peptide GP1 and, to a lesser extent, GP14, or to smaller peptides here contained in the longer ones such as NR1 NR2, or the sporozoites themselves (Pf). However, responses were lower and borderline (threshold of positivity = 2) in monkey M88 as compared with the 2 others. The PHA is a positive stimulation control.

In monkeys immunised only by GP1 adjuvanted by AS2, positive responses were mainly recorded in monkey M73 and were only borderline positive to the immunising peptide GP1 in monkey V114 (whereas they are essentially negative in monkey M51).

Figure 7 : Elispot assays

The figure shows responses recorded in monkey M73 and V114 receiving GP1 peptide and monkeys M88 and M91 receiving the mix of GP1 + GP14. The results are expressed as a mean of SFCs, i.e. of colonies secreting Interferon-g in an Elispot

assay. Results are strongly positive in all monkeys towards several peptides, e.g. the recombinant 729 and immunising peptides GP1 in M73, as well as *P. falciparum* sporozoites, most peptides or sporozoites employed in monkey V114, the recombinants 729 and NN for monkey M88 and M91 as well as the immunising peptide GP1 and, to a lesser extent GP14 in the same monkeys, as well as *P. falciparum* sporozoites in the same monkeys.

Figure 8 a-d: LDH levels

The figure shows the various levels of LDH detected in the various monkeys mentioned above, as compared to the controls (blue line). The horizontal line is the threshold of positivity determined as the mean OD value in controls + 3 standard the assay determined as the mean value given by uninfected aotus control blood + 3 standard deviations (results below this threshold value are negative and results above this threshold value are positive). The horizontal axis indicates the days following sporozoite injection, when samples were taken and processed in the DELI-LDH assay.

Example 4

Sequence data and supplementary information

The following further information exemplifying the invention is supplied:

Sequence Data - Gene: full Sequence (K1 parasite)

- Protein: full Sequence (K1 parasite)
- Clones DG729 / DG679 (T9/96 parasite)
- Note on LSA-3 sequence in parasite 3D7

Gene & Protein - Structure . Restriction map . Hydrophobicity

- Oligonucleotides employed
- Organisation

Regions & Comments - NR-A . R1 . R2 . NR-B . R3 . NR-C

Conservation - of the gene

- of the sequence
- of repeat region R2
- comparison of immunising and challenging sequences

Stage Specificity & Subcellular Location

Homologies - Intraspecies

- Interspecies

Synthetic Peptides & Recombinant Proteins used for Chimpanzee Immunisations

- Peptides CT1 . NR1 . NR2 . RE
- Recombinant proteins β -729. GST-729. GST-NN. GST-PC

Methods**References to Example 4**

Full sequence listings in the appropriate format are also provided herein.

SEQUENCE DATA

K1 PARASITE STRAIN- clone k1.2

Accession Nber AJ007010

GENE full sequence

	10	20	30	40	50	60	70	80	90	100
1	atgacaaata	gtaattacaa	atcaaaataat	aaaaatata	atgaaataaa	taattgaacaa	ataactacca	tatttaataag	aacaaatag	aactcgataa
101	aaaaatgtca	tatgagagaa	aaaaataata	agtaactttt	tttgatcdaa	attttgacat	gcaccatttt	aataatggct	gtacaatag	ataataacgt
201	agatgaaaa	actaaataat	aaatataaat	aaaaaataaa	aaaaaataaa	aaaaatcaac	tatatagtat	gtataatata	tatatatata	tatatatata
301	tatatatata	tatatattta	ttttttattta	ttttttaatt	tttttttttt	tatatattat	tttttagctg	atataaacaa	gagttggaaa	aaaaatcgt
401	atgtcgataa	gaaattgaat	aaactattta	acagaagttt	aggagaatct	caagtataatg	gtgaat tag	tagtgaagaa	gtaaaaggaaa	aaattcttga
501	cttattagaa	gaagggaata	catttaactga	aagtgtagat	gataatdaaa	atttagaaga	agccgaagat	ataaaggaaa	atatcttatt	aagtaataa
601	gaagaaacaa	aagaaataat	tattgacaat	ttattaataa	atatgggaca	aaattcgaaa	aaacaagaaa	gtgtatcaga	aaagtacaa	gcagtgatg
701	aactttttaa	tgaattatta	aatagtgtag	atgttaatgg	agaagtataa	gaaaatattt	tggaggaaa	tcaagttaat	gacgatattt	ttaatagttt
801	agtaaaaagt	gttcaacag	aacaacaaca	caatgttgaa	gaaaaagttg	agagaaggtt	agagaagaa	gacgaagaa	gtgtagaaga	aaatgtaga
901	gaaaatataa	agaaaaatga	gaaacgaagt	gtacgctcaa	gtgttcaaga	aagttatagct	tcaagtggtg	atgaaatgat	agattcaagt	attgaaagaa
1001	atgtagctcc	aactgttgaa	gaaatcgtag	ctccaaagtt	tgtagaagct	ctgggtccaa	gtgttgaa	aagttgaa	gaaatgtgtg	agagaaggtt
1101	agctcgaagt	gttgaaagaa	gtgtagctga	aaatgttgaa	gaaagtgtg	ctgaaatgt	tgaagaaagt	gtagctgaaa	atgttgaa	aactcgtgct
1201	ccaaactgtt	aggaatcgt	agctccaa	gttgaaagaa	ttgtactctc	aagtttgtta	gaaagtgtg	ctccaaagtt	tgaagaaagt	gtagaagaaa
1301	atgttcaaga	aagtgtagct	gaaatgtgtg	aggaaggtgt	agctgaaat	cttgaaagaa	gtgtagctga	aaatgttgaa	gaaagtgtg	ctgaaatgtg
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1801	ccaaactgtg	aggaaggtgt	aggaagaaat	gttgaaagaa	gtgtagctga	aaatgttgaa	gaaagtgtg	ctgaaatgt	tgaagaaagt	gtagctgaaa
1901	atgttcaaga	aagtgtagct	gaaatgtgtg	aggaatcgt	agctccaa	cttgaaagaa	tgttagctcc	aactgttgaa	gttagctga	ctccaaagtt
2001	tgttagctga	gtgttgaa	aggttgtaga	gaaatgtgtg	aggaaggtgt	agctgaaat	gttgaaagaa	gtgtagctga	aaatgttgaa	gttagctga
2101	gaaagtgtg	ctgaaatgt	tgaagaaagt	gttagctccaa	ctgttgaa	aactgttagct	ccaaactgtg	aggaatgtg	agctccaa	gttgtagaa
2201	gtgttagctga	aagtgtagct	gaaagtgtg	aggaatcgt	tgaagaaagt	gtagctgaaa	atgttgaa	aagtgtagct	gaaatgtgtg	aggaaggtgt
2301	agctcgaagt	gttgaaagaa	gtgtagctga	aaatgttgaa	gaaatcgtg	ctccaaactgt	tgaagaaagt	gtagctccaa	gtgttgtaga	aagttgtgct
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2501	atgttcaaga	aagtgtagct	gaaatgtgtg	aggaatcgt	agctccaa	cttgaaagaa	tgttagctcc	aagtgtaga	gaaagtgtg	ctccaaagtt
2601	tgaagaaagt	gtgttgaa	aggttgtaga	gaaatgtgtg	aggaaggtgt	agctgaaat	gttgaaagaa	gtgtagctga	aaatgttgaa	gttagctga
2701	ttaaattgaa	tagaagaa	aaaagaaat	gtagtcacca	caactactaga	aaactgtaga	gaaactacag	ctgaaagtgt	aactactttt	agtaacatct
2801	ttagggagat	acaagaaat	actattacta	atgatactat	agaggaaata	ttagaagaa	tccacgaa	tgtattaa	gcgctttg	aaaataccca
2901	aagtgaagag	gaaagaaag	aagtaataga	tgttaattga	gaagtaaa	aagaggtgc	taccacttta	atagaactg	tggaaacagg	agaaagaa
3001	agcgcaata	caattacga	aattattgaa	aatttagaag	aaaaatcag	agaaagtta	gaaatgttg	cagagaatt	agagaataa	aacgaactg
3101	tatttaatac	tgtattagat	aaagttagg	aaacagtga	aattagcgga	gaaagttag	aaaacaaatga	aatggataa	gcatttttta	gtgaaattg
3201	tgataatgta	aaaggaatac	aagaaatatt	atcaacaggt	atgttccgaa	ttagtagaac	cagtatagta	atccaatcag	aagaaaggt	tgtattgaat
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3601	gaaactgtta	gtattattga	agaaatggaa	gaaatattgt	ttagatgtatt	agaggaagaa	aaagaaagatt	tacacagaaa	gatgatagat	gcaatagaa
3701	aatccataga	aataatctta	gattctaaag	agaaactga	atctatttaa	gataaagaaa	aagatgttct	actagtgtt	gaagaaagtt	aagacaatga
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3901	gaagaaactc	aagagttaa	tgaagttaga	gcagatttaa	taaaagatat	ggaaaaatta	aaagaattag	aaaaagcatt	atcagaagat	tctaaagaaa
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4201	tataaagaat	taaaacctat	tgaacacagat	attttagaag	agaaaaaaga	aatagaaaaa	gatcattttg	aaaaattcg	agaaagagct	gaagaaataa
4301	aaagtcttga	agcagatata	ttaaaagag	tatcttcatt	agaaagttga	gaagaaaaaa	aatttagaaga	agtaacgaa	ttaaaagaag	aggttagaaca
4401	tataataagt	ggtgatgccc	atataaaaag	tttggaagaa	gatgattttg	aagaagttaga	tgaattttaa	ggaaatata	tagacatggt	aaagggagat
4501	atggaaatag	gggatatgga	taaggaaagt	ttagaagatg	taacaacaaa	acttggaaga	agagttgaat	cctttaaaga	tgttttatct	agtgcattag
4601	gcattggatga	agaaacaaatg	aaaacaagaa	aaaagctcaa	agaacctaa	ttggaaagag	tattatata	agagaggtt	aaagaagaa	caaagaaaaa
4701	ataaacaata	agaaagttaa	ggtttgatat	taaggataag	gaaccataag	atgaaatagt	agaagttaa	atgaaagata	aaagatacga	agaaagtgtg
4801	gaaagataa	tgaagaaaga	tataaadaa	gatgaagttg	agatataga	tgaagataa	gatgaagata	tgaatgaaga	caagatgaa	gttatagatt
4901	taatatgccca	aaaagagaaa	cgcattgaaa	aggttaaaag	gaaaaagaaa	aaattagaaa	aaaagttga	agaaaggtgt	agtggtctta	aaaacacgt
5001	agcgaagta	atgaaatag	ttcaaaaat	tgataaagaa	gttgataaag	aagtatctaa	agctttgaa	tcaaaaaatg	atgttactaa	tgttttaaaa
5101	caaaatcaag	atttttttag	taaaagttaa	aactctgtaa	aaaaatataa	agtatttgct	gcaccattca	tatctgcgt	tgaagcattt	gcataatag
5201	tagtgggttt	ttttacattt	tttttatttt	catcatgtgt	aacaatagct	tctcaactt	acttatatc	aaaagttgac	aaaactataa	ataaaataa
5301	ggagagaccg	tttcatctat	tgttatttga	tatcttttaa	aatttaaaac	attatttaca	acaaatgaaa	gaaaaattt	gtaaaagaaa	aaaataaat
5401	gtaatagaag	tacaacaaca	agctgagaaa	aaaggtaatg	tacaggtaac	aaataaaacc	gagaaaaaaa	ctaaagttag	taaaaataat	aaagtaccga
5501	aaaaaagaag	aacgcataaa	tcaaaaataa	5529						

Complete nucleotide sequence of the 5529 base-pair (bp) *lsa-3* gene. Bolded is a 168 bp intron; underlined are the 3 repeat regions R1, R2 and R3.

PROTEIN full sequence

```

1  MTNSNYKSNN KTYNENNNEQ ITTIFNRTNM NPIKKCHMRE KINKYFFLIK ILTCTILIWA VOYDNNSDIN KSWKKNTYVD
81  KKINKLFNRS LGESQVNGEL ASEEVKEKIL DLLEEGNTLT ESDVDNKNLE EAEDIKENIL LSNIEPKEN IIDNLLNNIG
161 QNSEKQESVS ENVQVSDELF NELLNSVDVN GEVKENILEE SQVNDIDFNS LVKSVQOEQQ HNVEEKVEES VEENDEESVE
241 ENVEENVEEN DGGSVASSVE ESTASSVDES IDSSIEENVA PTVEEIVAPS VVESVAPSVE ESVEENVEES VAENVEESVA
321 ENVEESVAEN VEESVAENVE EIVAPTVEEI VAPTVEEIVA PSVVESVAPS VEESVEENVE ESVAENVEES VAENVEESVA
401 ENVEESVAEN VEESVAENVE EIVAPTVEEI VAPTVEEIVA PSVVESVAPS VEESVEENVE ESVAENVEES VAENVEESVA
481 ENVEESVAEN VEESVAENVE ESVAENVEES VAENVEEIVA PTVEEIVAPT VEEIVAPSVV ESVAPSVEES VEENVEESVA
561 ENVEESVAEN VEESVAENVE ESVAENVEEI VAPTVEEIVA PTVEEIVAPS VVESVAPSVE ESVEENVEES VAENVEESVA
641 ENVEESVAEN VEEIVAPTVE EIVAPTVEEI VAPSVVESVA PSVEESVEEN VEESVAENVE ESVAENVEES VAENVEESVA
721 ENVEEIVAPT VEEIVAPTVE EIVAPSVVES VAPSVVESVA ENVEESVAEN VEESVAENVE ESVAENVEES VAPTVEEIVA
801 PSVEESVAPS VEESVAENVA TNLSDNLLSN LLGGIETEEI KDSILNEIEE VKENVVTIL ENVEETTAES VTTFNILEE
881 IQENTITNDT IEEKLEELHE NVLSAALENT QSEBEKKEVI DVIEEVKEEV ATTLIETVEQ AEEKSANTIT EIFENLEENA
961 VESNENVAEN LEKLNETVFN TVLDKVEETV EISGESLENN EMDKAFSEI FDNVKGIQEN LLTGMFRSIE TSIVIQSEK
1041 VDLNENVVSS ILDNIENMKE GLLNKLENIS STEGVQETVT EHVEQNVYVD VDPVPMKDQF LGILNEAGGL KEMFFNLEDV
1121 FKSESDVITV EEIKDEPVQK EVEKETVSII EEMENIVDV LEEEEKDLTD KMIDAVEESI EISSDSKEET BSIKDKKDV
1201 SLVVEEVQDN DMDESVEKVL ELKNMREELM KDAVEINDIT SKLIEETQEL NEVEADLIK MEKLELEKA LSEDSKEIID
1281 AKDDTLEKVI EEEHDITTL DEVVELKDV EDKIEKVSIL KDLEEDILKE VKEIKELESE ILEDYKELKT IETDILEEKK
1361 EIEKDFEFK EEEAEIKDL EADILKEVSS LEVVEEKKLE EVHELKEEVE HIISGDAHIK GLEEDDLEEV DDLKGSILDM
1441 LKGDMELGDM DKESLEDVTT KLGERSVSLK DVLSSALGMD BEQMKTRKKA QRPKLEEVLL KEEVKKEPKK KITKKKVRFD
1521 IKDKPKDEI VEVEMKDEDI EEDVEEDIEE DIEEDKVEDI DEDIDEDIGE DKDEVIDLIV QKEKRIEKVK AKKKKLEKKV
1601 EEGVSGLKHH VDEVMKYVQK IDKEVDKEVS KALESKNDVT NVLKQNDQFF SKVKNFVKKY KVFAAPFISA VAAFASYVVG
1681 FFTFSLFSSC SKVDKTINKN KERPFYSFVF DIFKNLKHVL QQMKEKFSKE KNNNVIETIN KAEKKGNVQV
1761 TNKTEKTTKV DKNKVKPKR RTQKSKZ 1786

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Complete peptide sequence of the 1786 amino-acid (aa) LSA-3 protein. Bolded are 3 potential start sites; underlined are the 3 repeat regions R1, R2 and R3.

T9/96 PARASITE CLONE

Accession Nber AJ007011

Nucleotide sequence

```

1' agtgatgaac tttttaatga attattaaat agttagatg ttaattggaga agtaaaagaa aatatttttg aggaaagtca
81' agttaatgac gatattttta atagtttagt aaaaagtgtt caacaagaac aacaacacaa tggttgaagaa aaagttgaag
161' aaagtgtaga agaaaatgac gaagaaagtg tagaagaaaa tgtagaagaa aatgtagaag aaaatgacga cggaagtgt
241' gctccaagt ttgaagaaag tataagcttca agtgttgatg aaagtataga ttcaagtatt gaagaaaatg tagctccaac
321' tggtgaagaa atcgtagctc caactgttga agaaattgta gctccaagt ttgtagaag tggtgctcca agtgttgaag
401' aaagtgtagc tccaagtgtt gaagaaagt tagctgaaaa tggtgaagaa agtgtagctg aaaatgttga agaaatcgt
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561' tggtgaagaa agtgtagctg aaaatgttga agaaagtgt gctgaagaa ttgaagaaat cgtagctcca actgttgaag
641' aaagtgtagc tccaagtgtt gaagaaattg tagctccaac tggtgaagaa agtgtagctc caactgttga agaaattgta
721' gttccaagt ttgaagaaag tgtagctcca agtgttgaag aaagtgtagc tgaaaatgtt gaagaaagt tagctgaaaa
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1121' tattagagga gatacaagaa aatactatta ctaagtatac tatagaggaa aaattagaag aactccacga aaatgtatta
1201' agtgcgcgt tagaaaatac ccaaagtga gaggaaaaga aagaagtaat agatgtaatt gaagaagtaa aagaagaggt
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1361' aagaaaatgc agtagaaagt aatgaaaagt ttgcagagaa tttagagaaa ttaaacgaaa ctgtatttaa tactgtatta
1441' gataaagtag aggaacacgt agaaattagc ggagaaagtt tagaaaacaa tgaaatggat aaagcatttt ttagtgaat
1521' atttgataat gtaaaaggaa tacaagaaaa tttatttaaca ggtatgtttc gaagtataga aaccagtata gtaatccaat
1601' cagaagaaaa ggttgatttg aatgaaaatg tggttagttc gatttttagat aatatagaaa atatgaaaga aggtttatta
1681' aataaattag aaaatatttc aagtactgaa gg 1712'

```

Partial nucleotide sequence of the *lsa-3* gene in the Thai parasite clone T9/96. Bolded is the sequence of insert DG729. Insert DG679, the largest among the LSA-3 insert family (see text of the present article and Guérin-Marchand *et al.*, 1987), spans from nucl. 32' to nucl. 1712'. Underlined are the adjacent repeat regions R1 and R2. Position 1' corresponds to nucl. 694 in the original K1 sequence.

Peptide sequence

```

1'  SDELFNELLN SVDVNGEVKE NILEESQVND DIFNSLVKSV QQEQQHNVEE KVESVEEND EESVEENVEE NVEENDDGSV
81'  ASSVEESIAS SVDESIDSSI EENVAPTVEE IVAPTVEEIV APSVVESVAP SVEESVAPSV EESVAENVEE SVAENVEEIV
161' APSVEESVAE NVEESVAENV EESVAENVEE SVAENVEESV AENVEEIVAP TVEESVAPT VEE EETVAPTVEE SVAPTVEEIV
241' VPSVEESVAP SVEESVAENV EESVAENVEE SVAENVEESV AENVEESVAE NVEEIVAPSV EETVAPTVEE SVAENVATNL
321' SDNLLSNLLG GIETEEIKDS ILNEIEEVKE NVVTTILEKV EETTAESVTT FSNILEEIQE NTITNDTIRE KLEELHENVL
401' SAALENTQSE EEKKEVIDVI EEVKKEEVATT LIETVEQABE ESESTITBIF ENLEENAVES NEKVAENLEK LNETVFNTVL
481' DKVEETVEIS GESLENNEMD KAFFSEIFDN VKGIQENLLT GMFRSIETSI VIQSEEKVDL NENNVSSILD NIENMKEGLL
561' NKLENISSTE 570'

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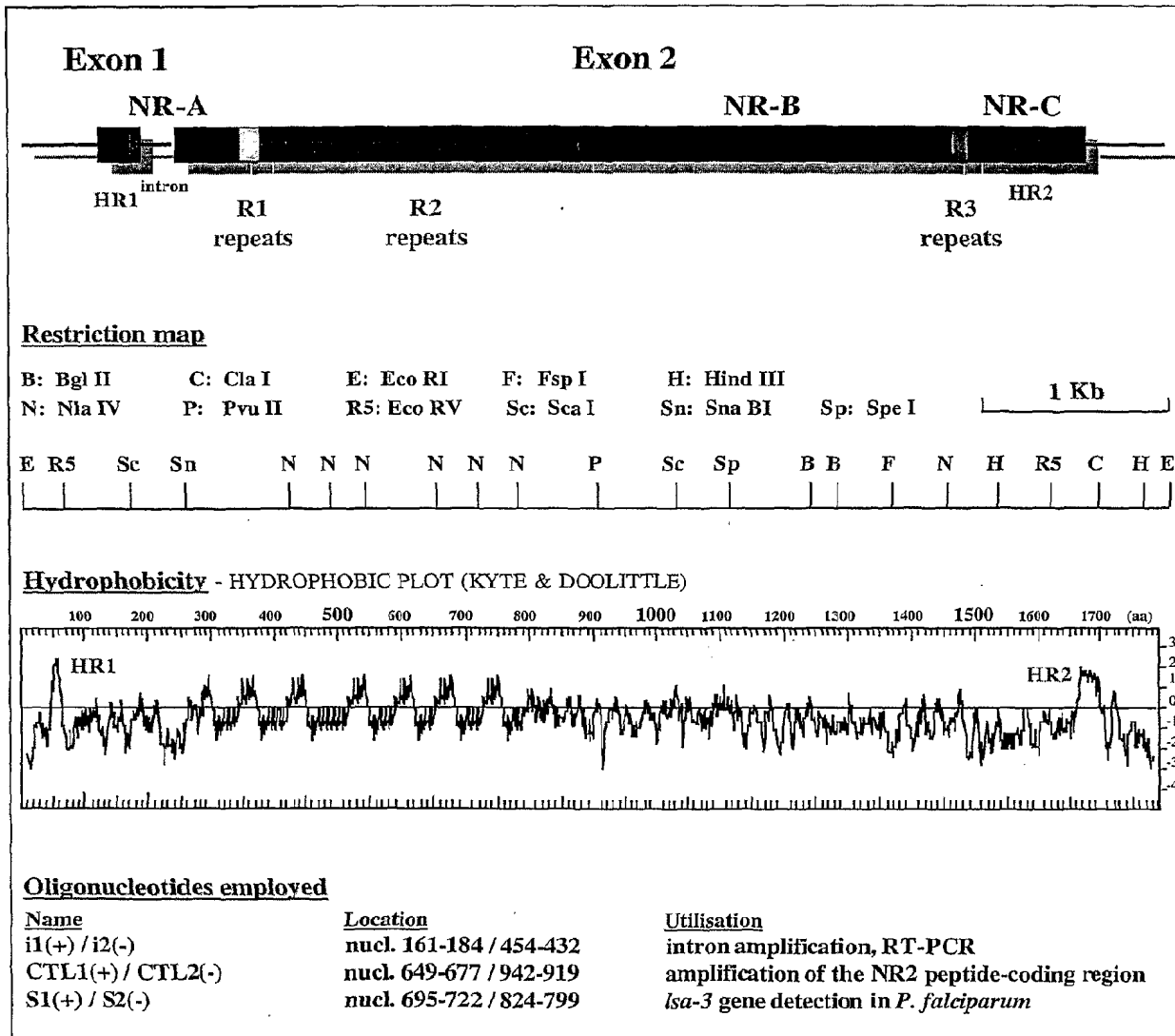
Partial peptide sequence of the LSA-3 protein in the Thai parasite clone T9/96. Bolded is the sequence of insert DG729. Insert DG679, the largest among the LSA-3 insert family (see text of the present article and Guérin-Marchand *et al.*, 1987), spans from aa 12' to aa 570'. Underlined are the 2 adjacent repeat regions R1 and R2. Position 1' corresponds to aa 176 in the original K1 sequence.

Note on LSA-3 sequence in parasite 3D7

The *lsa-3* gene sequence in parasite clone 3D7 (derived from strain NF54 used in the present article for chimpanzee challenges) is found in the complete sequence of *P. falciparum* Chromosome 2 (Gardner *et al.*, 1998) where it was annotated as *resa-h3* (Accession Number AE001424).

LSA-3 GENE & PROTEIN

K1 Parasite Strain - clone k1.2



Gene [5529 bp]

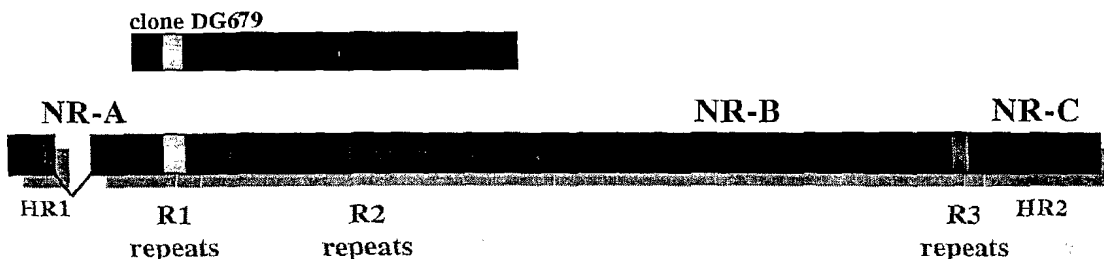
Regions	Length	Location	Regions	Length	Location
NR-A	834 bp	nucl. 1-834	R2 repeats	1623 bp	nucl. 1003-2625
Exon 1	198 bp	nucl. 1-198	NR-B	2148 bp	nucl. 2626-4773
Intron	168 bp	nucl. 199-366	R3 repeats	126 bp	nucl. 4774-4899
Exon 2	5164 bp	nucl. 367-5529	NR-C	630 bp	nucl. 4900-5529
R1 repeats	168 bp	nucl. 835-1002			

Protein [786 amino acids - Predicted MW : 200 kDa]

<u>Regions</u>	<u>Length</u>	<u>Location</u>	
NR-A	278 aa	aa 1-278	Non-repeated region A
HR1	18 aa	aa 46-63	Hydrophobic region 1: putative signal peptide
R1	56 aa	aa 223-278	Conserved repeat region
R2	541 aa	aa 279-819	Polymorphic repeat region
NR-B	716 aa	aa 820-1535	Non-repeated region B
R3	42 aa	aa 1536-1577	Conserved repeat region
NR-C	210 aa	aa 1578-1786	Non-repeated region C
HR2	33 aa	aa 1662-1694	Hydrophobic region 2: putative transmembrane domain

REGIONS & COMMENTS

k1.2 and T9/96 clones



NR-A

1 MTNSNYKSNN KTYNENNNEQ ITTFNRTNM NPIKKCHMRE KINKYFFLIK ILTCTILIWA VQYDNNSDIN
 71 KSWKKNTYVD KKLNKLFNRS LGESQVNGEL ASEEVKEKIL DLLEEGNTLT ESVDDNKNLE EAEDIKENIL
 141 LSNIEBPKEN IIDNLLNNIG QNSEKQESVS ENVQVSDELF NELLNSVDVN GEVKENILEE SOVND DIFNS
 211 LVKSVQEQEQ HN 222

Underlined and bolded are the 3 potential start sites; in green is a stretch of 17 uncharged and hydrophobic residues (HR1), preceeded and followed by two short positively charged regions. As confirmed by the combined neural approach documented in Nielsen *et al.* (1997): 1) this constitutes a potential signal sequence peptide, consistent with the subcellular location of LSA-3 in sporozoites and in liver forms, 2) most likely cleavage site is located between aa 63 and 64. Underlined is the NR2 peptide-coding region which shows a perfect conservation among *P. falciparum* parasites.

R1

223 VEEK VEES VEEN DEES VEEN VEEN VEEN DDGS VASS VEES IASS VDES IDSS IEEN 278

R1 is distinguished from region R2 by its specific tetrapeptide motifs and an extremely high conservation in T9/96 (100% at both nucleotidic and peptidic levels) and 3D7 (1 point mutation over 168bp/56aa, see sequence AE001424 in Gardner *et al.*, 1998) parasite clones.

R2 / k1.2 clone

279	<u>VAPT</u>	<u>VEEIVAPS</u>	<u>VVESVAPS</u>	<u>VEESVEEN</u>		
307	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>		
339	<u>VEEIVAPT</u>	<u>VEEIVAPT</u>	<u>VEEIVAPS</u>	<u>VVESVAPS</u>	<u>VEESVEEN</u>	
379	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>	
419	<u>VEEIVAPT</u>	<u>VEEIVAPT</u>	<u>VEEIVAPS</u>	<u>VVESVAPS</u>	<u>VEESVEEN</u>	
459	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u> <u>VEESVAEN</u>
515	<u>VEEIVAPT</u>	<u>VEEIVAPT</u>	<u>VEEIVAPS</u>	<u>VVESVAPS</u>	<u>VEESVEEN</u>	
555	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>		
587	<u>VEEIVAPT</u>	<u>VEEIVAPT</u>	<u>VEEIVAPS</u>	<u>VVESVAPS</u>	<u>VEESVEEN</u>	
627	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>			
651	<u>VEEIVAPT</u>	<u>VEEIVAPT</u>	<u>VEEIVAPS</u>	<u>VVESVAPS</u>	<u>VEESVEEN</u>	
691	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>		
723	<u>VEEIVAPT</u>	<u>VEEIVAPT</u>	<u>VEEIVAPS</u>	<u>VVESVAPS</u>	<u>VEESVEEN</u>	
763	<u>VEESVAEN</u>	<u>VEESVAEN</u>	<u>VEESVAEN</u>			
787	<u>VEESVAPT</u>	<u>VEEIVAPS</u>	<u>VEESVAPS</u>			
811	<u>VEESVAEN</u>	818				

R2 / T9/96 clone

104' VAPT VEEIVAPT VEEIVAPS VVESVAPS VEESVAPS
 140' **VEESVAEN VEESVAEN**
 156' VEEIVAPS
 164' **VEESVAEN VEESVAEN VEESVAEN VEESVAEN VEESVAEN**
 204' VEEIVAPT VEESVAPT VEEIVAPT VEESVAPT VEEIVVPS VVESVAPS
 252' **VEESVAEN VEESVAEN VEESVAEN VEESVAEN VEESVAEN**
 292' VEEIVAPS VEEIVAPT
 308' **VEESVAEN 315'**

Bolded are stretches of tandemly repeated and conserved octapeptides VEESVAEN which can vary in number, from 2 to 7 in both strains. Underlined are the highly conserved 40 aa repeated blocks which separate these stretches in strain K1. In clone T9/96, no particular organization is observed in R2. This region is nevertheless composed of similar and conserved tetrapeptides compared to strain K1, except one variant VVPS which is specific for T9/96.

NR-B

819 VA TNLSDNLLSN LLGGIETEEI KDSILNEIEE VKENVVTIIL ENVEETTAES VTTFSNILEE
 881 IOENTITNDT IEEKLEELHE NVLSAALENT QSEBEKKEVI DVIEEVKEBV ATTLIETVEO AEEKSANTIT
 951 EIFENLEENA VESNENVAEN LEKLNETVFN TVLDKVEETV EISGESLENN EMDKAPFSEI FDNVKGIQEN
 1021 LLTGMPRSIE TSIVIOSEK VDLNENVVSS ILDNIENMKE GLLNKLENIS STEGVQETVT EHVEQNVYVD
 1091 VDVPAMKDQF LGILNEAGGL KEMFFNLEDV FKESDVTITV EEIKDEFVQK EVEKETVSII EEMENIVDV
 1161 LEEEKEDLTD KMIDAVEESI EISSDSKEET ESIKDKEKDV SLVVEEVQDN DMDESVEKVL ELKNMEELM
 1231 KDAVEINDIT SKLIETQEL NEVEADLIK MEKLKELEKA LSDSKEIID AKDDTLEKVI EEHDITTTIL
 1301 DEVVELKDVE EDKIEKVDL KDLEEDILKE VKEIKELESE ILEDYKELKT IETDILEEKK EIEKDHFEKF
 1371 EEEAEBIKDL EADILKEVSS LEVVEEKKLE EVHELKEEVE HIISGDAHIK GLEEDDLEEV DDLKGSILDM
 1441 LKGDMEIGDM DKESLEDVTT KLGERVESLK DVLSSALGMD EEQMKTRKKA QRPKLEEVLL KEEVKEEPPK
 1511 KITRKKVFRD IKDK EEKDDT EMEM 1535

Underlined is the partial NR-B region of insert DG679 (parasite clone T9/96) which shows a high degree of conservation with K1 sequences and contains only 6 bp substitutions leading to 5 aa mutations (bolded). Shaded is the highly conserved HLA-B53 restricted epitope Ia90 identified by Aidoo *et al.* (2000).

R3

1536 KDED IEED VEED IEED IEED KVED IDDED IDDED IGED KDEV ID 1577

The same regular spacing of the hydrophobic isoleucine and valine residues is observed in region R3 which is predicted, according to its HCP analysis (not shown), to adopt an α -helical conformation and is preceded by a cluster of helix-breakers (proline) alternating with β -sheet segments. This region also shows a high degree of conservation with LSA-3 sequences in clone 3D7 (see sequence AE001424 in Gardner *et al.*, 1998) and in isolates from various geographical origins (Daubersies, P. *et al.*, in preparation).

NR-C

1578 LIV QKEKRIEKVK AKKKKLEKKV EECVSGLKKH VDEVMKYVQK IDKEVDKEVS KALESKNDVT
 1641 NVLKQNDQFF SKVKNFVKKY **KVFAAPFISA** **VAAFAGYVVG** **FFTFSLFSSC** **VTIASSTYLL** SKVDKTINKN
 1711 KERPFYSFVF DIFKNLKHYL QMKKEKFSKE KNNNVIEVTN KAEKKGNVQV TNKTEKTKV DKNNKVPKKR
 1781 RTQKSKZ 1786

Bolded (and in green) is a second hydrophobic region (HR2) which could constitute a transmembrane domain, consistent with the subcellular location of the antigen in sporozoites and in liver forms.

CONSERVATION

Conservation of the gene

LSA-3 gene and protein detected in 100 % of *P. falciparum* parasites by:

• **PCR ANALYSIS** performed with S1(+) / S2(-) primers on:

- 70 isolates from Ivory Coast, Madagascar, Myanmar, Brazil and Columbia
- 12 Thai sporozoite strains
- 6 laboratory strains or clones (K1, T9/96, NF54, Palo Alto, 150, 3D7)
- 23 Senegalese isolates - Data published in Bottius *et al.* (1996) [where clone DG157 is a member of the LSA-3 clone family and encodes for a part of region NR-A]

The expected 130 bp amplification product was found in the 111 samples

• **IFAT** performed with anti-NR2 peptide and anti-GST-PC antibodies (mouse and chimpanzee sera) on:

- 30 Thai sporozoite strains
- 2 infected liver sections: one from a *Cebus* (day 5 post-challenge) and one from a chimpanzee (day 6 post-challenge)

**Detection in the 32 samples
100 % of positive parasites in each assay**

Conservation of the sequence

Data published in Ben Mohamed *et al.* (1997)

Direct PCR sequencing performed with CTL1(+) / CTL2(-) primers from nucl. 740 to nucl. 861 (122 bp) on:
 . 5 strains or clones (K1, T9/96, NF54, Palo Alto, 3D7)
 . 7 African, 5 Brazilian, 3 Malagasi, 3 Burmese isolates
 . 5 Thai clones (Drulhe *et al.*, 1998)

100 % bp conservation in 28 samples
for:

NR2 peptide-
coding region



NR-A R1

R2

NR-B

R3 NR-C

clone DG679

T9/96

100%
(0/104aa)

100%
(0/56aa)

98%
(5/255aa)

3D7

99.6%
(1/278aa)

98.2%
(1/56aa)

99.6%
(3/716aa)

99%
(2/210aa)
90.5%
(4/42aa)

Data published in Aidoo *et al.* (2000)

From nucl. 4741 to nucl. 4767 (27 bp):
 1 silent mutation in 12/18 Gambian isolates
 [nucl. 4746/codon 1526: cca -> ccc]

97.5 % conservation in nucleotides
100 % conservation in amino acids
for:

HLA-B53 restricted
la90 CTL epitope

Homology at amino acid level (nber of mutation(s)/length of the region analysed) in parasite clones T9/96 and 3D7

Precise position and description of bp/aa mutations in parasite K1, T9/96 and 3D7 is detailed in 2 tables from section "comparison of immunising and challenging sequences". Conservation of the polymorphic repeat region R2 is analysed in the following section.

Conservation of the repeat region R2

Conservation of R2 motif sequences

MOTIFS		<i>P. FALCIPARUM</i> LINES		
PEPTIDIC	NUCLEOTIDIC	K1	T9/96	3D7
VAEN	gta gct gaa aat --t --- --- --c	30/31 1/31	12/13 1/13	9/10 1/10
VAPS	gta gct cca agt --g --- --- ---	9/16 7/16	5/6 1/6	16/17 1/17
VAPT	gta gct cca act --- --- --- --a	14/14 -	7/7 -	7/9 2/9
VEES	gtt gaa gaa agt	42/42	17/17	15/15
VEEI	gtt gaa gaa atc --- --- --- --t	13/20 7/20	5/8 3/8	16/22 6/22
VEEN	gta gaa gaa aat	11/11	-	1/1
VVES	gtt gta gaa agt --c --- --- ---	7/7 -	- -	- 1/1
VVPS	gta gtt cca agt	-	1/1	-
VVPT	gta gtt cca act	-	-	2/2

Peptide and nucleotide sequence comparison of R2 tetrapeptidic motifs between K1, T9/96 and 3D7 parasites. Although the organization of these tetrapeptide motifs varies within R2 (see section "regions & comments" for K1, and T9/96 and see sequence AE001424 in Gardner *et al.* (1998) for 3D7), conservation of their sequences remains extremely high (e.g. only 3 strain specific tetrapeptides (VVPS, VVPT) among a total of 231 motifs and no single nucleotide mutation in the 74 VEES, 21 VAPT, 12 VEEN motifs).

Conservation of R2 helicity

HYDROPHOBIC CLUSTER PLOT: regions NR-A/R1/R2 in clone k1.2 (parasite stain K1, aa184-457)



HYDROPHOBIC CLUSTER PLOT: regions NR-A/R1/R2 in clone DG679 (parasite clone T9/96, aa 21'-284')



Prediction of LSA-3 conformation (K1 strain) by hydrophobic cluster plot (HCP) analysis (for symbols, see Gaboriaud *et al.*, 1987) reveals a regular organization of the R1-R2 repeat regions, in a succession of α -helical stretches interrupted by the helix-breaker proline residues (tetrapeptides VAPT). This α -helical conformation is also strongly suggested by the remarkable regular spacing, every 4 residues, of the hydrophobic valine throughout the entire R1-R2 block, i.e. 597 aa. To a lesser extent, the same regular spacing of the hydrophobic isoleucine and valine residues is observed in the R3 repeat region which is predicted, according to its HCP (not shown), to adopt an α -helical conformation and is preceded by a cluster of helix-breakers (proline) alternating with β -sheet segments.

Regions R1-R2 from T9/96 shows a different organization since sequences separating the stretches of tandemly repeated octapeptide VEESVAEN consist of a mosaic of various tetrapeptides also found in blocks R1-R2 of clone k1.2. Nevertheless and according to its HCP, the secondary structure of R1-R2 seems perfectly conserved in T9/96 compared to K1, with the same succession of α -helical stretches interrupted by the proline helix-breaker residues. This result is strongly suggestive of important structural constraints at least on this part of the protein.

Conservation of R2 conformation

Antibodies	recombinant proteins and peptides (ELISA)		NF54 sporozoites (IFAT)
	from K1	from T9/96	
anti-RE (T9/96)	+ / GST-NN	+ / GST-729	+
anti-GST-NN (K1)	+ / GST-NN	+ / RE	+

As shown in this table, conservation of R2 conformation is suggested by the constant recognition of recombinant proteins and peptides (K1 and T9/96 derived sequences) in ELISA and of NF54 sporozoites in IFAT by anti-RE (T9/96) or anti-GST-NN (K1) antibodies (mouse sera and human immunopurified antibodies).

Comparison between immunising and challenging sequences

Mutations identified and localisation

LSA-3 Regions ¹	Clones ²	Mutated nucleotide ³	Mutated codon ³	Original K1 sequence ⁴	Mutated sequence ⁴
NR-A (1-834)	3D7	191	64	gat (D)	gct (A)
R1 (835-1002)	3D7	926	253	gga (G)	gct (E)
NR-B (2626-4773)	T9/96	2754	852	aac (N)	aaa (K)
	T9/96	2796	876	aac (N)	aat <i>sil.</i>
	3D7 + T9/96	2998	944	aag (K)	gag (E)
	T9/96	3005	946	gca (A)	gag (E)
	3D7 + T9/96	3008	947	aat (N)	agt (S)
	T9/96	3066	956	aat (N)	aaa (K)
	3D7	3972	1268	gaa (E)	gag <i>sil.</i>
	3D7	4546	1460	aca (T)	gca (A)
R3 (4774-4899)	3D7	4650	1494	aag (K)	aaa <i>sil.</i>
	3D7	4791	1541	gaa (E)	gat (D)
	3D7	4798	1544	gta (V)	ata (I)
	3D7	4810	1548	ata (I)	gta (V)
NR-C (4900-5529)	3D7	4870-71	1567-68	-	12 bp ins. ⁵
	3D7	4940	1591	gcg (A)	gag (E)
	3D7	5508	1780	aga (R)	agt (S)

Position in the reference *lsa-3* gene (strain K1) and description of the mutations identified in parasites clones T9/96 and 3D7 (which was originally cloned from strain NF54 and is considered here as representative of NF54 for complete comparison purposes). As reported in section "conservation of the sequence", NR2 peptide-coding region of the NF54 strain used for the chimpanzee challenges was found 100 % homologous to K1 sequence.

1. Comments on region R2 from K1, T9/96 and 3D7 parasites are given in the preceding section. Numbers in brackets define first and last nucleotides of the corresponding region in strain K1. 2. 3D7 sequences analysed here cover the entire gene and were defined by compiling data from 3 different sources: 1) construct VR2555 which contains a PCR-amplified truncated *lsa-3* gene (nucl. 432-5095; P. Daubersies, unpublished data), 2) construct VR2556 which contains a full-length PCR-amplified LSA-3 cDNA (Hoffman S., personal communication), 3) *lsa-3* gene sequence identified in *P. falciparum* Chromosome 2 (seq. AE001424 in Gardner *et al.*, 1998). Mutations were considered as such if they were observed in at least 2 out of 3 sequences. 3. Numbers for mutated nucleotides and codons correspond to their location in the reference *lsa-3* gene and protein respectively (in strain K1). 4. Original and mutated codons are followed in brackets with the corresponding amino acid (one-letter code). 5. 12 base pair insertion "gaagatatagat", leading to a 4 amino acid insertion "EDID".

Correspondences and homologies

LSA-3 regions		LSA-3 sequences ¹					
		in strain K1		in clone T9/96		in clone 3D7	
		sequenced	immunis. ²	sequenced	immunis. ³	sequenced ⁴	challenge ⁵
NR-A	length in base pairs	834	60 (CT1)	316	141 (GST-729)	834	60 + 141
	location in gene	1-834	586-645	519-834	694-834	1-834	386-645 + 694-834
	length in amino acids	278	20	104	47	278	20 + 47
	location in protein	1-278	140-159	119-222	176-222	1-278	140-159 + 176-222
	nucleotid. mutation(s)			0	0	1	0
R1	length in base pairs	168	-	168	168 (GST-729)	168	168
	location in gene	835-1002	-	835-1002	835-1002	835-1002	835-1002
	length in amino acids	56	-	56	56	56	56
	location in protein	223-278	-	223-278	223-278	223-278	223-278
	nucleotid. mutation(s)			0	0	1	1
R2⁶	length in base pairs	1623	240 (GST-NN)	636 (full seq.)	141 (GST-729)	924 (full seq.)	924
	location in gene	1003-2625	1269-1509				
	length in amino acids	541	80	212	47	308	308
	location in protein	279-819	369-448				
	nucleotid. mutation(s)						
NR-B	length in base pairs	2148	2006 (GST-PC)	764	-	2148	2009
	location in gene	2626-4773	2769-4773	2626-3389	-	2626-4773	2769-4773
	length in amino acids	716	667	255	-	716	667
	location in protein	820-1535	869-1535	820-1074	-	820-1535	869-1535
	nucleotid. mutations)			6		5	5
R3	length in base pairs	126	126 (GST-PC)	-	-	126	126
	location in gene	4774-4899	4774-4899	-	-	4774-4899	4774-4899
	length in amino acids	42	42	-	-	42	42
	location in protein	1536-1577	1536-1577	-	-	1536-1577	1536-1577
	nucleotid. mutations					4	4
NR-C	length in base pairs	630	630 (GST-PC)	-	-	630	630
	location in gene	4900-5529	4900-5529	-	-	4900-5529	4900-5529
	length in amino acids	210	210	-	-	210	210
	location in protein	1578-1786	1578-1785	-	-	1578-1786	1578-1786
	nucleotid. mutations					2	2
Non-repeated regions [NR-A, -B, -C]	total length in bp/aa	3612/1204	2695/898	1080/360		3612/1204	2836/944
	total nber nucl./aa mut. nucl./aa homology (%)			6/5 99.4/98.6		8/6 99.8/99.5	7/5 99.8/99.5
Conserved regions [NR-A, -B, -C, R1, R3]	total length in bp/aa	3906/1302	2821/940	1248/416		3906/1302	3130/1042
	total nber nucl./aa mut. nucl./aa homology (%)			6/5 99.5/98.8		13/11 99.7/99.1	12/10 99.6/99.0

Definition and comparison of immunising and challenging sequences. As in the preceeding table, *lsa-3* sequence in clone 3D7 (originally cloned from NF54 strain) is considered here as representative of the actual NF54 strain used for sporozoite challenges.

1: All sequence locations (bp and aa) correspond to the reference numbering in *lsa-3* gene and protein from strain K1. **2:** Immunising sequences in strain K1 correspond to peptide CT1 and recombinant proteins GST-NN and GST-PC. **3:** Immunising sequences in clone T9/96 correspond to peptides NR1, NR2, and RE and recombinant protein GST-729 from which these 3 peptides were derived. **4:** See note (2) in the preceeding table. **5:** Challenging sequences are defined as 3D7 sequences corresponding to cumulated immunising sequences from both K1 and T9/96 parasites. **6:** A more detailed analysis of R2 is given in the preceeding section. Due to length polymorphism, numbering in region R2 is non-relevant in parasites other than K1. Lengths given for T9/96 and 3D7 correspond to their respective fully sequenced region R2.

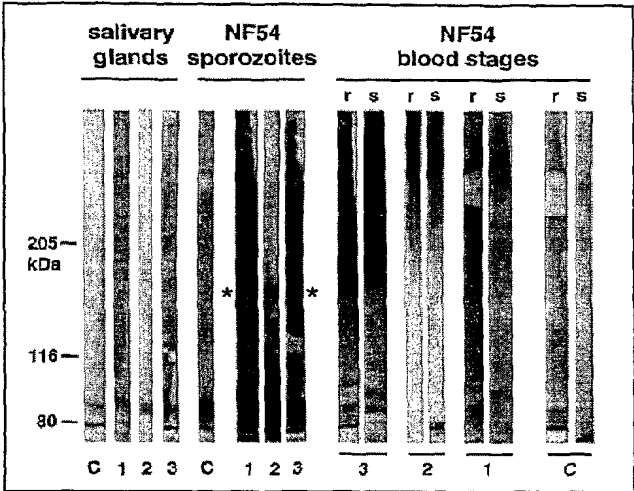
**STAGE SPECIFICITY
&
SUBCELLULAR LOCATION**

SPOROZOITES

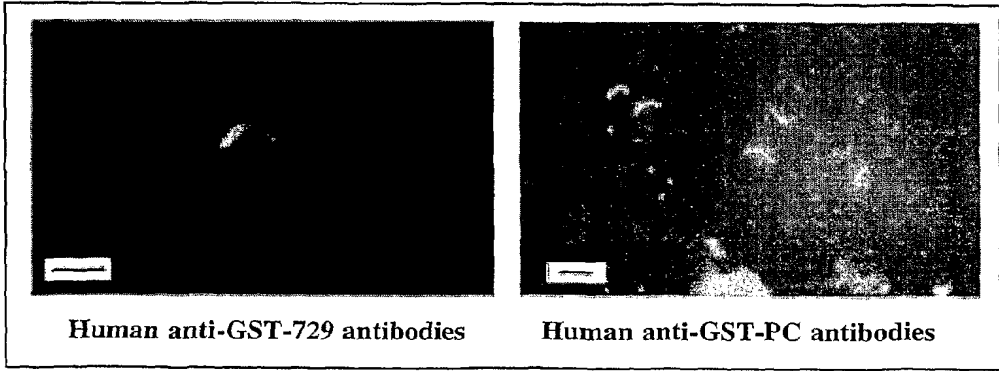
A. RT-PCR



B. WESTERN BLOT ANALYSIS

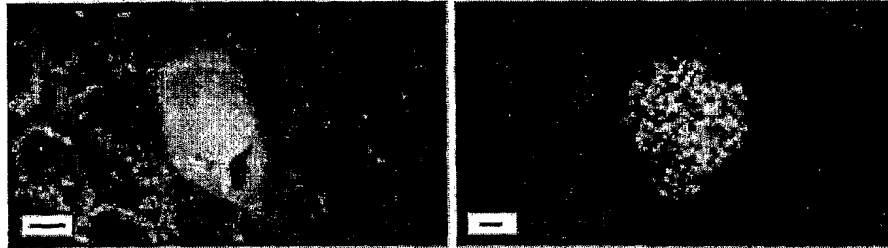


C. Immunofluorescence Antibody Test



A. Due to the difficulties in obtaining an adequate quantity of sporozoite mRNA, Northern blot analysis could not be performed at this stage and transcription of *lsa-3* gene was studied by RT-PCR. Oligonucleotides i1 (+) and i2 (-), located 3' of exon 1 and 5' of exon 2 respectively, allowed amplification of the expected 125 bp fragment in NF54 mRNA (lane 2) whereas control DNA (lane 3) and contaminating DNA (lane 2) gave a 293 bp band. Lanes 1: 100 bp ladder (Amersham). Effective splicing of the intron were further confirmed by subcloning of the 125 bp fragment and complete sequencing.

B. Western blot analysis of protein extracts from uninfected mosquito salivary glands, NF54 sporozoites and blood stages (r: rings, s: schizonts) using mouse antisera directed against C) control GST recombinant protein, 1) GST-PC recombinant protein, 2) oligonucleotides GP5-GP6-GP8-GP11, 3) GST-729 recombinant protein (see Methods). In sporozoites, LSA-3 is visualized as a 175 kDa protein (*), in agreement with LSA-3 theoretical molecular weight calculated (for NF54 sequence) with the PEPTIDEMASS program (Wilkins *et al.*, 1997 and <http://www.expasy.ch/tools/peptide-mass.html>). **C.** By IFAT, LSA-3 appears to be located in some areas of the membrane and to distribute over the cytoplasm of sporozoites. Bars correspond to 10 μ m.

LIVER STAGE**Immunofluorescence Antibody Test**

mouse anti-NR2 peptide antibodies
Five day-old liver schizonts (*Cebus*)

chimpanzee anti-NR2 peptide antibodies
Six day-old liver schizonts (chimpanzee)

By IFAT, LSA-3 appears located in the parasitophorous vacuole of trophozoites and in the pseudocytomere, i.e. the fluffy material surrounding merozoites from mature liver schizonts. Bars correspond to 20 μ m.

RT-PCR, Northern blot, Western Blot: not accessible

BLOOD STAGE**Northern blot**

. negative (DNA probes: DG729 and PC insert; data not shown)

Western blot (see sporozoite Western blot for comparison with sporozoite and control extracts run in parallel)

- . negative on extracts from all forms when using mouse antisera directed against peptides GP5-GP6-GP8-GP11 (see Methods) and GST-PC recombinant protein
- . cross-reactions observed on ring and schizont extracts when using human and/or mouse antibodies directed against R2 repeats (anti-GST-729, -GST-NN and -RE antibodies)

IFAT

- . negative on all blood stage forms with antibodies against NR2 peptide and GST-PC recombinant (not shown)
- . cross-reactions observed on rings and schizonts with human and mouse antibodies directed against R1-R2 repeats (anti-GST-729, -GST-NN and -RE antibodies; not shown)

HOMOLOGIES

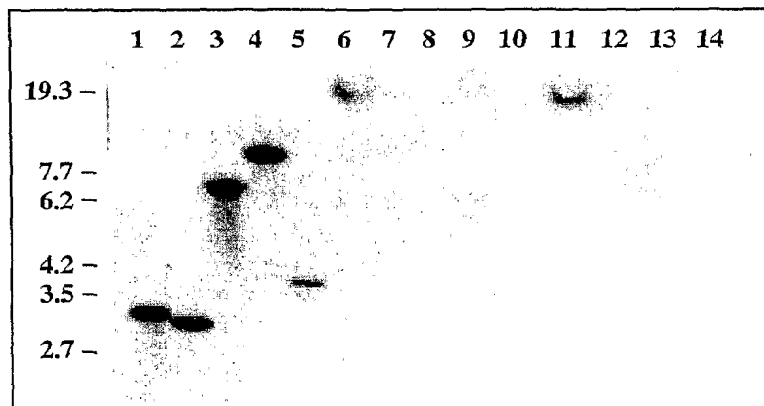
INTRASPECIES / INTERSPECIES

Data banks screening (GenBank, EMBL and SwissProt) with LSA-3 non-repeated sequences did not reveal any significant homology (>30%) with other known genes or proteins. As expected from their high valine and glutamine content, R2 repeated sequences did show significant homologies with PfRESA repeats, expressed at the surface of infected-erythrocytes and a member of the *P. falciparum* glutamic acid-rich antigenic network which also comprises antigens Pf11.1 and Ag332 (Moelans & Schoenmakers, 1992).

lsa-3 gene is a single-copy gene in *P. falciparum* genome.

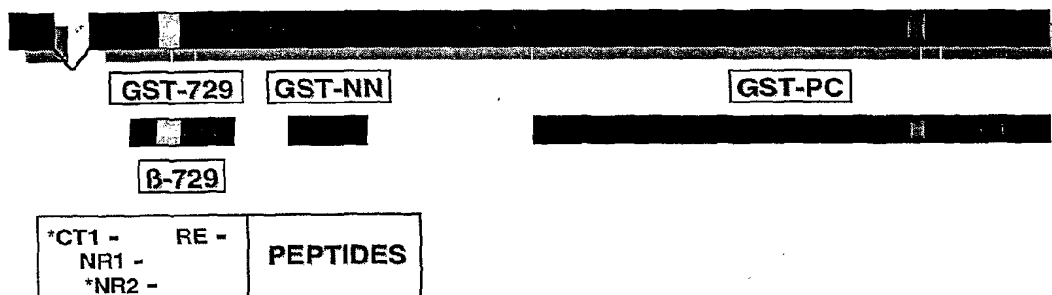
A single band, corresponding to a single-copy gene, is observed below in each of lanes 1-4 where a DG729 DNA probe was hybridized at low-stringency with (see section "Restriction map") *Sca* I/*Eco* RI (lanes 1-2) and *Eco* RI/*Hind* III (lanes 3-4)-digested *P. falciparum* DNA from NF54 (lanes 1, 3) and T9/96 (lanes 2, 4) parasites.

The same experiment performed with *Eco* RI/*Hind* III-digested DNA from *Plasmodium gallinaceum* (lane 7), *vivax* (lane 8), *knowlesi* (lane 9), *cynomolgi* (lane 10), *chabaudi* (lane 12), *yoelii* (lane 13) and *berghei* (lane 14) did not reveal any homologous sequences in these species, except in the simian parasite *P. reichenowi* (lane 5) which is closely related to *P. falciparum*. Lanes 6 and 11: molecular weight markers.



SYNTHETIC PEPTIDES & RECOMBINANT PROTEINS

used for chimpanzee immunisations



SYNTHETIC PEPTIDES

. CT1	aa 140-159	LLSNIEEPKENIIDNLLNNI
. NR1	aa 177-201	DELFNELLNSVDVNGEVKENILEES
. NR2	aa 198-223	LEESQVNDDIFNSLVKSVQQEQQHN
. RE	derived from block R2 of clone DG729	VESVAPSVEESVAPSVEESVAENVEESV

*: for the immunisations, CT1 and NR2 were also employed as palmitoyl-conjugated lipopeptides prepared as described in Ben Mohamed *et al.* (1997).

RECOMBINANT PROTEINS

. **β-729**: β-galactosidase fused protein encoded by the DG729 insert (aa 1'-150'), cloned in frame within the *Eco* RI site of the phage λgt11

. **GST-729**: GST (glutathione-S-transferase)-fused protein encoded by the DG729 insert (aa 1'-150'), cloned in frame within the *Eco* RI site of pGEX.A plasmid (Invitrogen)

. **GST-NN**: GST-fused protein (aa 369-447) encoded by the klenow filled-in *Nla*IV-*Nla*IV restriction fragment (nucl.1269-1509, K1 strain), cloned in frame within the *Sma*I site of pGEX-2T plasmid (Invitrogen)

. **GST-PC**: GST-fused protein (aa 869-1786) encoded by the klenow filled-in *Pvu*II-*Cla*I restriction fragment (nucl.2768-5574, K1 strain), cloned in frame within the klenow filled-in *Eco* RI site of pGEX-3X plasmid (Invitrogen)

METHODS

1. Parasites
2. Nucleic acid isolation and hybridisation
3. Cloning and sequencing protocols
4. PCR and RT-PCR amplifications
5. Peptides synthesis and production of recombinant proteins
6. Antibodies and antisera
7. Western blot analysis
8. Immunofluorescence Antibody Test

1. Parasites

Blood stages of *P. falciparum* T9/96 clone (Thaithong *et al.*, 1984), NF54 (Ponnudurai *et al.*, 19881) and K1 (Thaithong and Beale, 1981) strains were cultured as described by Trager and Jensen (1976). *P. falciparum* sporozoites were obtained from NF54 strain as described in Ponnodurai *et al.* (1989) and from mosquitoes fed with gametocytes produced *in vitro* from Thai patient isolates (Galey *et al.*, 1990). *P. falciparum* liver schizonts were identified in liver biopsies of a Sapajou monkey (*Cebus apella*, in day 5 post-sporozoite challenge) infected with the African isolate 730XI (Druilhe *et al.*, 1984), and of a chimpanzee (*Pan troglodytes*, in day 6 post-sporozoite challenge) infected with NF54 strain (Meis *et al.*, 1990).

2. Nucleic acid isolation and hybridisation

Parasite genomic DNA was purified from saponin-lysed infected erythrocytes (Robson *et al.*, 1991). Total RNA from sporozoites and parasite blood stages were extracted according to Chomczynski *et al.* (1987). DNA probes were randomly [³²P]-radiolabelled according to the manufacturer's recommendations (Amersham, UK). Southern and Northern blottings, probe hybridisations and washes were performed on 5-10 µg of material by standard methods (Sambrook *et al.*, 1989).

Low stringency cross-species hybridisations were performed overnight at 54°C in: 5x Denhardt's solution, 6x SSC buffer, 0.1 % SDS, 0.1 mg/ml sonicated salmon sperm DNA. Membranes were washed 30 min. at 54°C in 0.2X or 0.1X SSC buffer before autoradiography.

3. Cloning and sequencing protocols

A size-selected (0.5-1.5 Kb) genomic expression library was prepared in the phage λgt11 from *P. falciparum* T9/96 DNA and differentially screened with various stage-restricted sera as previously described (Guérin-Marchand *et al.*, 1987). λgt11-DG729 and -DG679 DNA were prepared from a liquid phage lysate. The gel-purified *Eco*RI inserts were cloned into plasmid pUC18 and sequenced. The DG729 insert was randomly radiolabelled and used as a probe to screen an *Eco*RI-digested genomic DNA library prepared from the K1 strain in the phage λgt10 (generously provided by G. Langsley, Pasteur Institute). Five positive clones were isolated and analysed. One of them, clone k1.2, was found to contain the largest *Eco*RI insert and was therefore chosen for subcloning and complete sequence analysis. This 6.7 Kb *Eco*RI fragment and subclones derived from it (spanning the entire insert) were cloned into pUC18. A series of Exonuclease III-digested subclones from the 1.8 Kb repeated regions R1-R2 of clone k1.2 was obtained using the Erase-a-Base Kit (Promega, U.S.A.). All clones and subclones described above were sequenced on both strands with insert flanking or internal oligonucleotidic primers using the dideoxy method (Sanger *et al.*, 1977) and the Sequenase enzyme system (United States Biochemicals Corp.).

4. PCR and RT-PCR amplifications

RT-PCR experiments were performed on 300-500 ng of total RNA (for blood stage parasites) or on the RNA pellet obtained from 10^6 - 10^7 NF54 sporozoites. cDNA were synthesized from 30 pmoles of primers S2(-) by the MMLV-reverse transcriptase in a final volume of 20 μ l according to the manufacturer's recommendations (Gibco-BRL). PCR reactions were carried out on 10 μ l of cDNA synthesis reaction or on 1 μ g of genomic DNA, according to the manufacturer's recommendations (Amersham, UK).

For *Isa-3* amplification in human blood samples and *P. falciparum* detection in challenged chimpanzees, PCR was performed as described in Bottius *et al.* (1996) where primers described within for clone DG157 correspond to primers S1 and S2 reported here.

5. Peptides synthesis and production of recombinant proteins

Peptides and lipopeptides used for chimpanzee immunisations were synthesized as described in Ben Mohamed *et al.* (1997). All peptides and lipopeptides were purified over 90% by reversed-phase chromatography, the impurities essentially consisting in shorter sequences. Long synthetic peptides GP5 (aa 1241-1346), GP6 (aa 1143-1255), GP8 (aa 1026-1095) and GP11 (aa 840-907) were synthesized as described in Roggero *et al.* (1995); they are all located in region NR-B (strain K1); i.e. the non-repeated region of PC insert.

Recombinant protein β -729 was prepared from a liquide lysate as described in Guérin-Marchand *et al.* (1987). Control GST protein and GST-fused recombinant proteins were prepared according to the manufacturer's recommendations (Invitrogen) except for GST-PC which was prepared from 20 liter cultures due to poor production yields. This large scale culture was incubated until $OD_{600} = 8.0$; bacteria were then pelleted, lysed using a French Press and filtered before standard purification.

6. Antibodies and antisera

Human antibodies were immunopurified on recombinant proteins and peptides as previously described in Marchand & Druilhe (1990) and Brahimi *et al.* (1993), respectively. Mouse and chimpanzee anti-NR2 peptide antibodies were induced respectively in mice and in chimpanzee Gerda by lipopeptide NR2 injections as described in Ben Mohamed *et al.* (1997). Mouse antisera against GST-PC recombinant protein and long peptides GP5-6-8-11 (used for Western blotting) were obtained following 3 subcutaneous injections of the immunogen (100 μ g) emulsified in SBAS2 adjuvant (Stoute *et al.*, 1997).

7. Western blot analysis

Proteins from intraerythrocytic parasites and sporozoites were solubilized in sodium dodecyl sulphate (SDS)-containing sample buffer, subjected to 5% SDS-polyacrylamide gel electrophoresis under reducing conditions, electroblotted onto nitrocellulose membrane and detected as described previously (Bouharoun-Tayoun & Druilhe, 1992), using mouse antibodies (at dilution 1/100). Visualisation was performed by peroxidase-conjugated goat anti-human IgG and chemoluminescence (ECL Western blotting reagents, Amersham).

8. Immunofluorescence Antibody Test (IFAT)

IFAT were performed as described previously (Druilhe *et al.*, 1986) on asynchronous erythrocytic cultures of *P. falciparum* NF54 strain, on freshly dissected live sporozoites labelled in suspension, on wet sporozoites deposited on poly-L-lysine-coated slides and on glutaraldehyde-fixed sporozoites, as well as on Carnoy-fixed liver schizonts. Positive IFAT on liver schizonts were verified by phase contrast microscopy and subsequent Giemsa staining of the sections (Druilhe *et al.*, 1984).

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Claims

- 1 A vaccine composition comprising a Th1-inducing adjuvant in combination with a protecting Liver Stage Antigen or immunological fragment thereof of a human malaria parasite with the proviso that when the immunological fragment is an immunological fragment of LSA-3, the Th1-inducing adjuvant is not Montanide.
- 2 A vaccine composition as claimed in claim 1 wherein the human malaria parasite is *Plasmodium falciparum*.
- 3 A vaccine composition as claimed in claim 1 or claim 2 in which the Th1-inducing adjuvant comprises either (a) QS21, De-O-acylated monophosphoryl lipid A (3D-MPL) and an oil in water emulsion wherein the oil in water emulsion has the following composition: a metabolisable oil, such a squalene, alpha tocopherol and tween 80; or (b) a vesicular adjuvant formulation comprising cholesterol, a saponin and optionally an LPS derivative.
- 4 A vaccine composition as claimed in claim 1 or 2 or claim 3 wherein said protecting Liver Stage Antigen is the Liver Stage Antigen 3 (LSA-3) or immunological fragment thereof.
- 5 A vaccine composition according to any one of claims 1 to 4 comprising in addition at least one other protecting antigen or an immunological fragment thereof, of a malaria parasite.
- 6 A vaccine composition as claimed in claim 4 in which the other malaria antigen is selected from the following group:
 - a) a hybrid protein comprising substantially all the C-terminal portion of the CS protein, four or more tandem repeats of the immunodominant region, and the surface antigen from hepatitis B virus (HBsAg), in particular RTS,S, or immunogenic derivatives including fragments thereof;
 - b) the TRAP protein of the T9/96 isolate of *Plasmodium falciparum* and proteins having at least 80% homology thereto and immunogenic derivatives including fragments thereof ;

- c) the MSP-1 of *Plasmodium falciparum* or *Plasmodium vivax* and proteins having at least 80% homology thereto and immunogenic derivatives including fragments thereof; and
- d) the MSP-3 of *Plasmodium falciparum* or *Plasmodium vivax* and proteins having at least 70% homology with the C-terminal region thereof, and immunogenic derivatives including fragments thereof.

- 7 A vaccine composition according to claims 1 to 6 capable of involving a T cell response in a mammal to the antigen or antigenic composition
- 8 A vaccine composition according to claims 1 to 7 capable of stimulating interferon γ production.
- 9 A vaccine composition according to claims 1 to 8, wherein the ratio of QS21:3D-MPL is from 1:10 to 10:1.
- 10 A vaccine composition according to claims 1 to 8, wherein the ratio of QS21:3D-MPL is from 1:1 to 1:2.5.
- 11 A process to make a vaccine composition according to any one of claims 1 to 10 comprising admixing QS21, 3D-MPL and the oil in water emulsion as defined in claim 2 with a protecting Liver Stage Antigen of a human malaria parasite.
- 12 A process according to claim 11 wherein the Liver Stage Antigen is LSA-3 of *Plasmodium falciparum* or immunological fragment thereof.
- 13 Use of a composition according to any one of claims 1 to 10 for the prophylaxis or treatment of malaria infections.

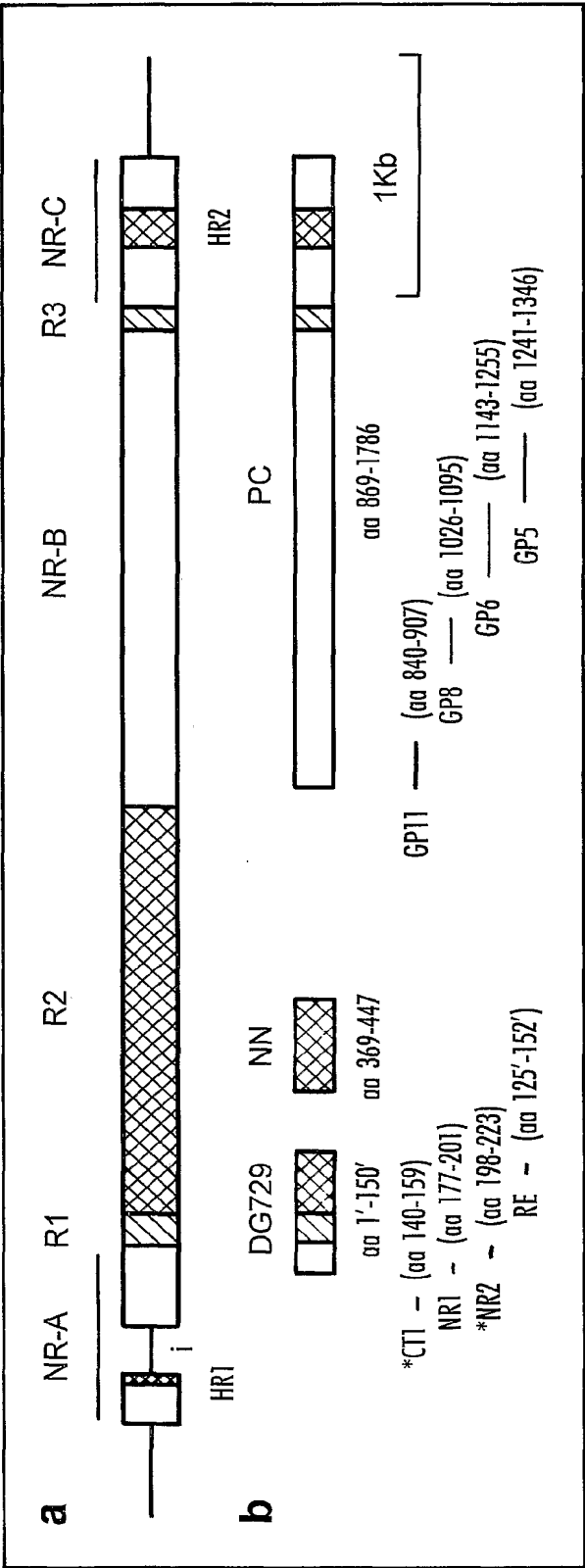
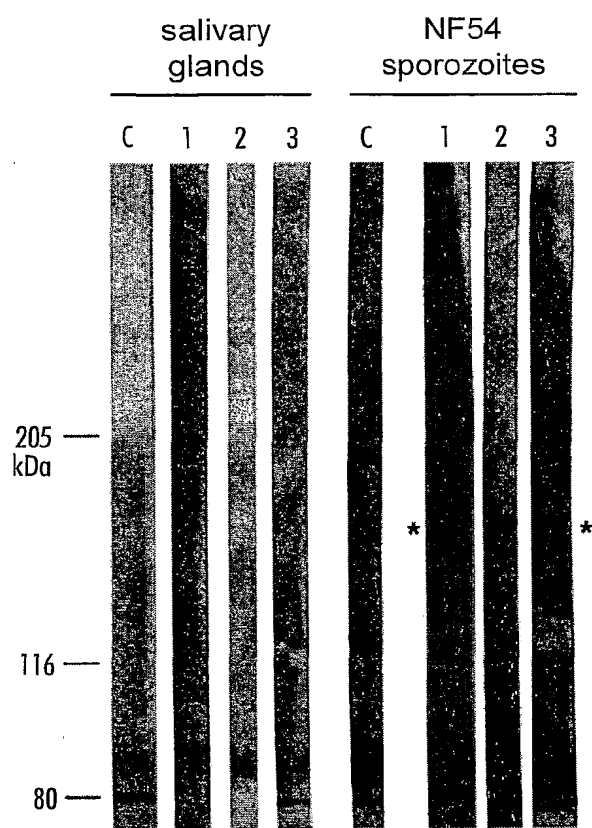


Fig. 1

2/11

**Fig. 2**

3/11

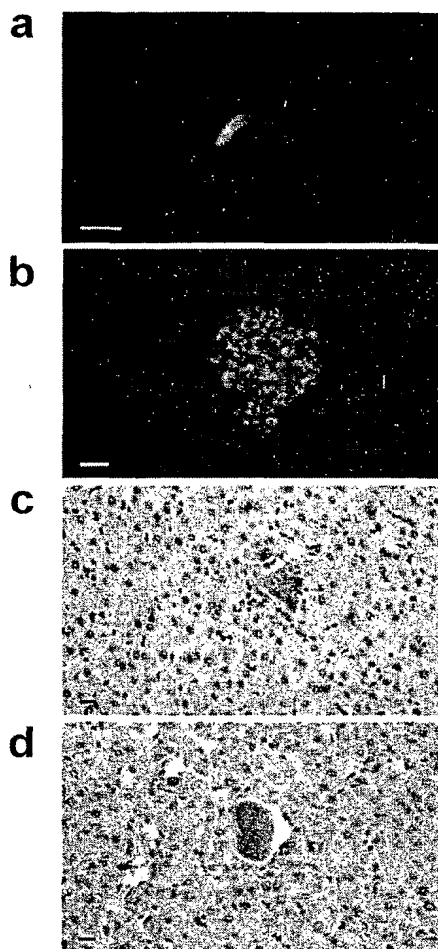


Fig. 3

4/11

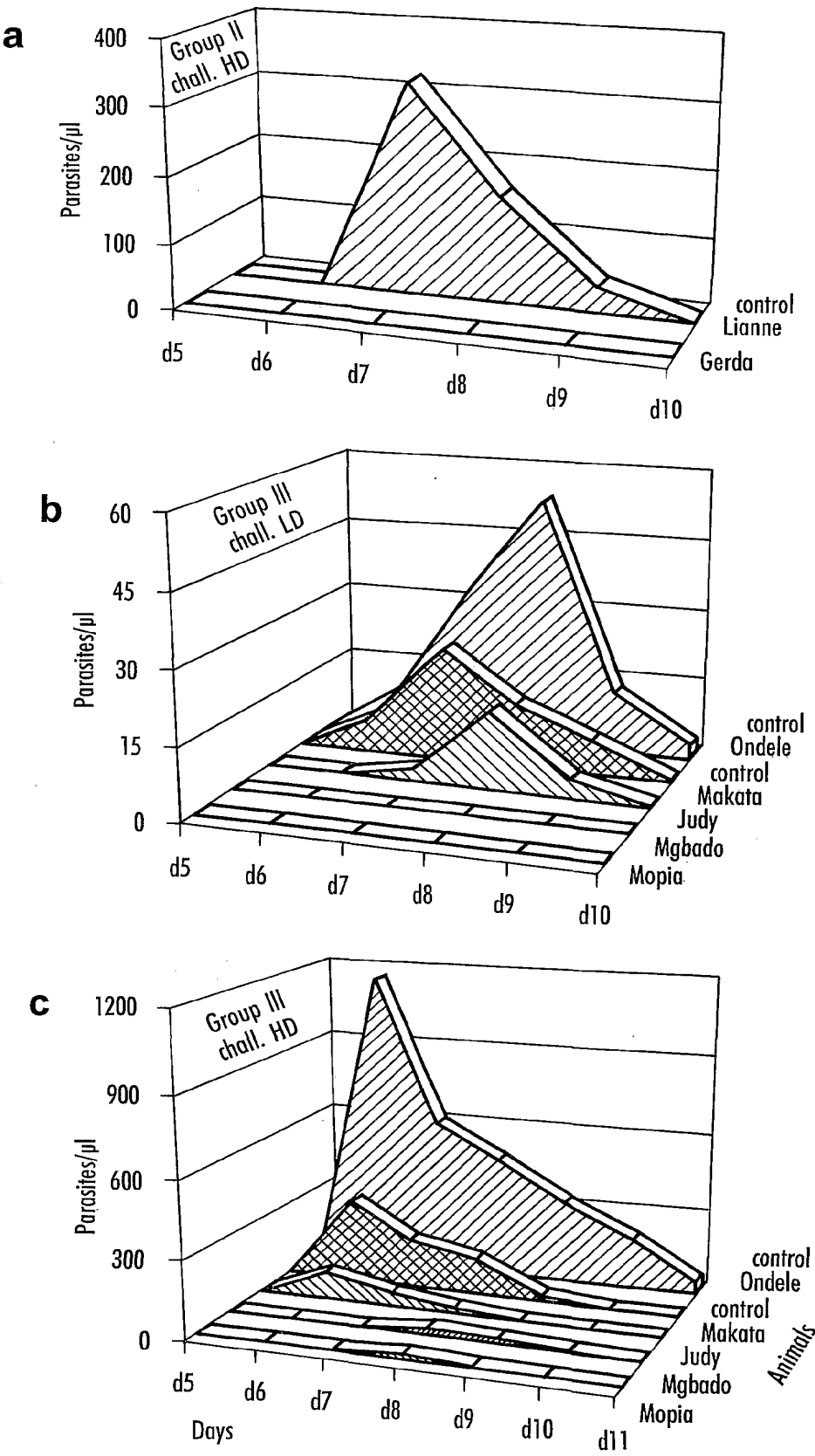


Fig. 4

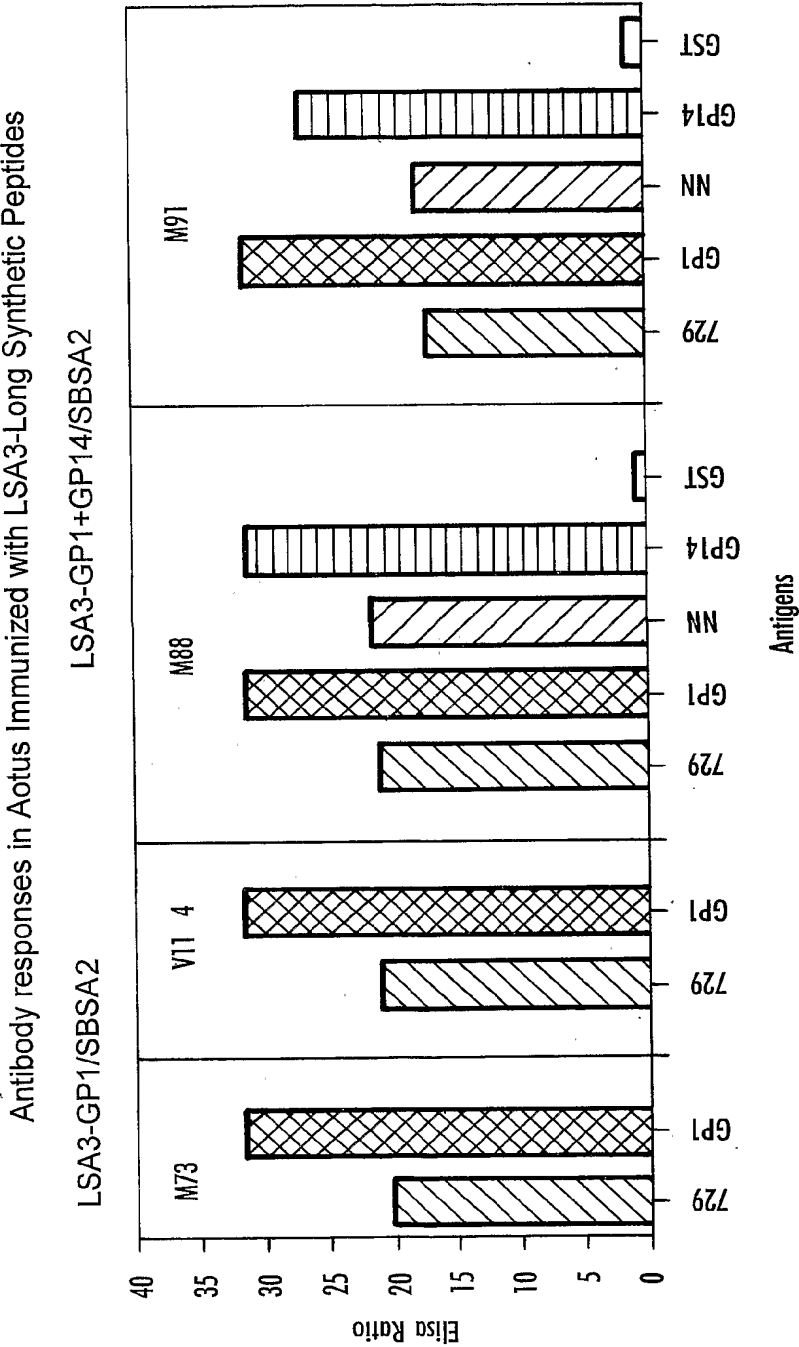


Fig. 5

6/11

Aotus monkeys immunized with GP1+GP14/SBSA2 Long Synthetic Peptides

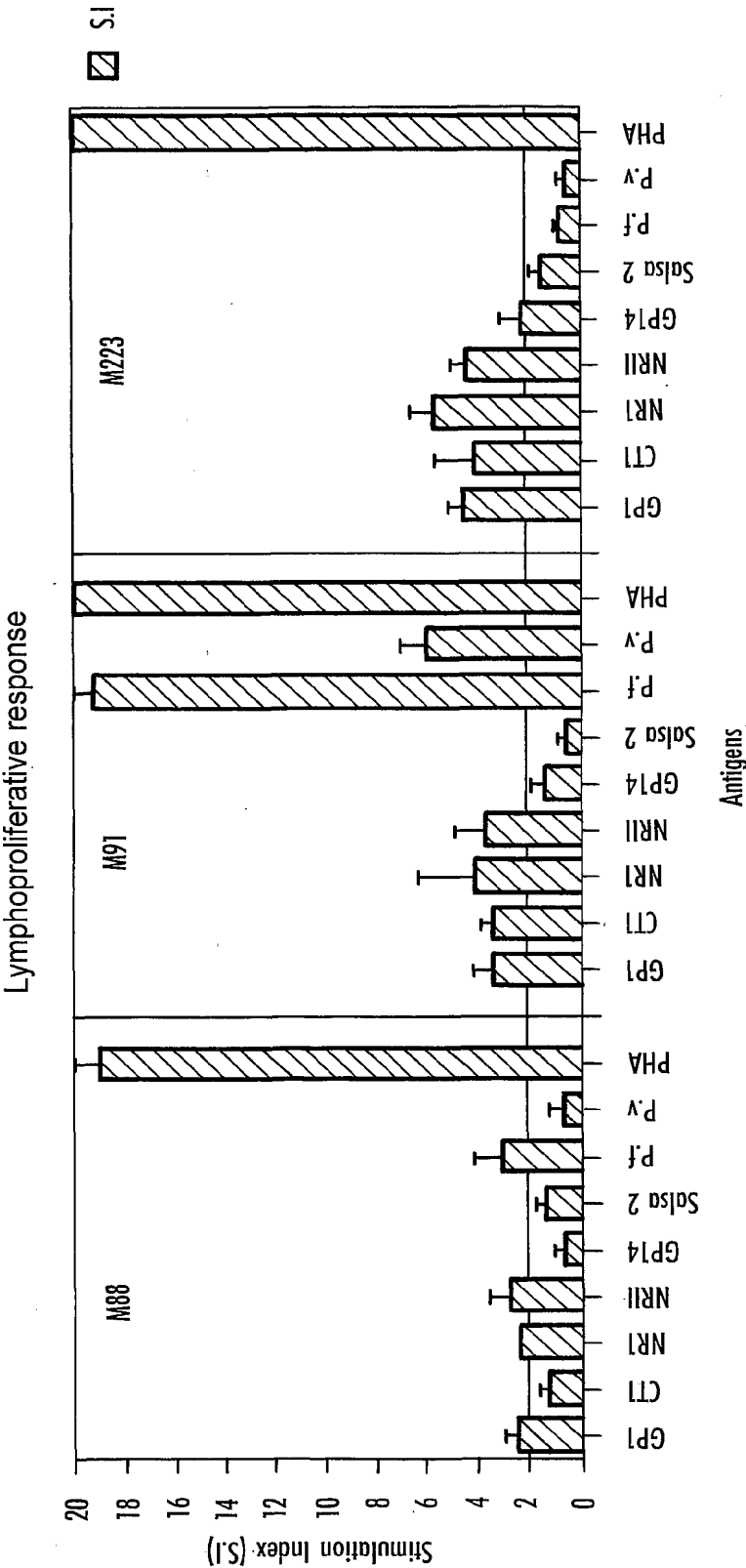


Fig. 6

Aotus immunized with LSA3-Long Synthetic Peptides

Elispot for IFN- γ

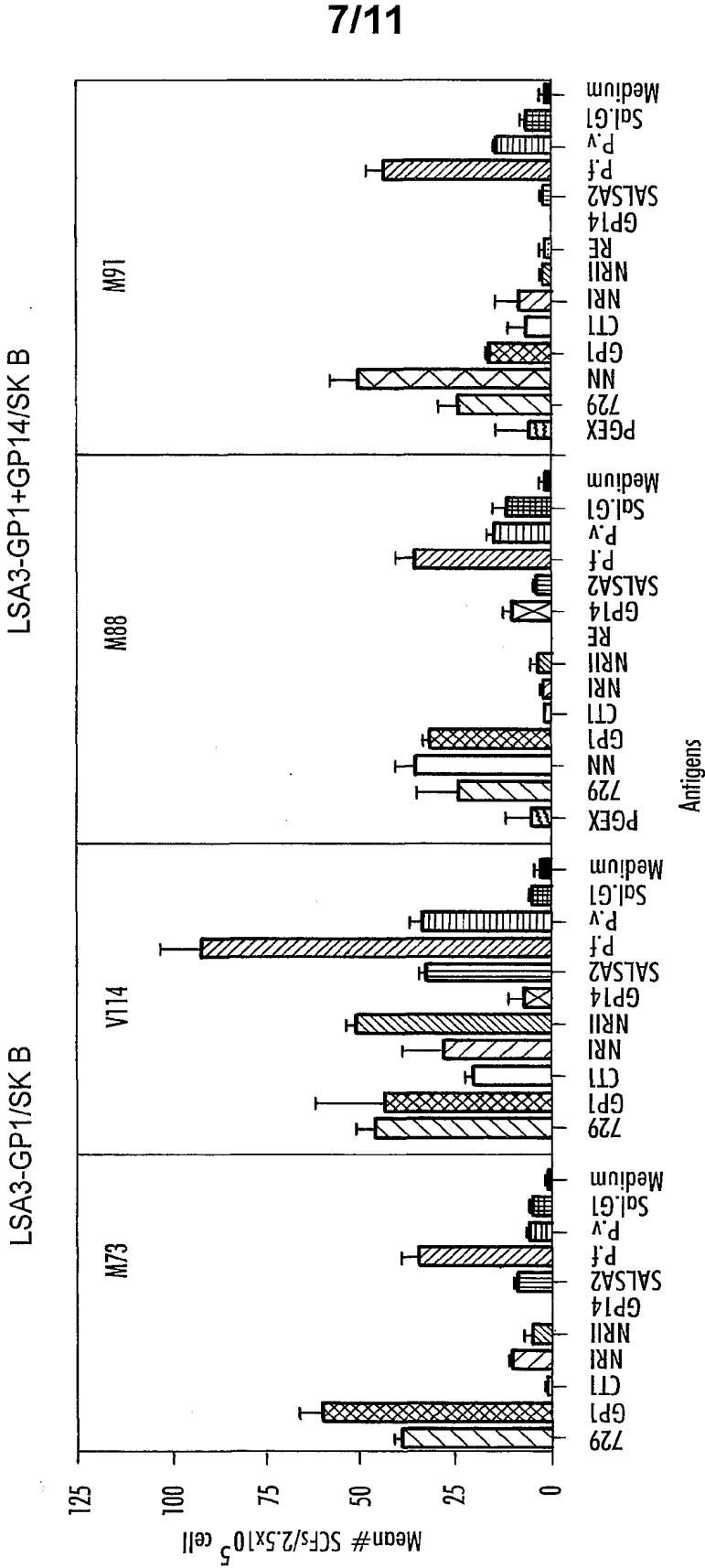
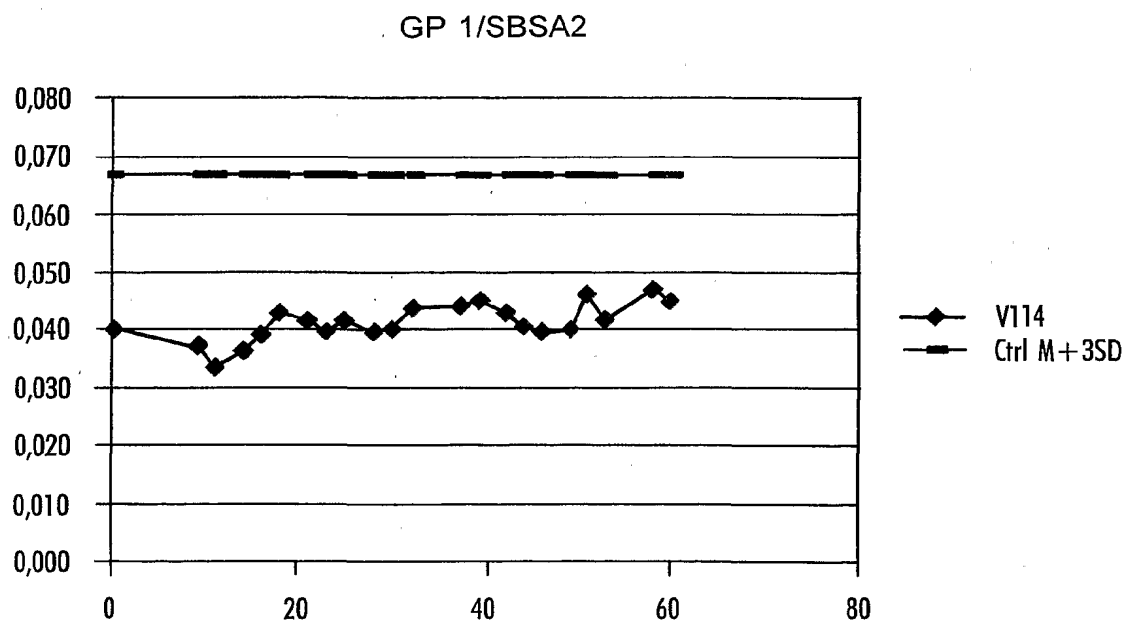
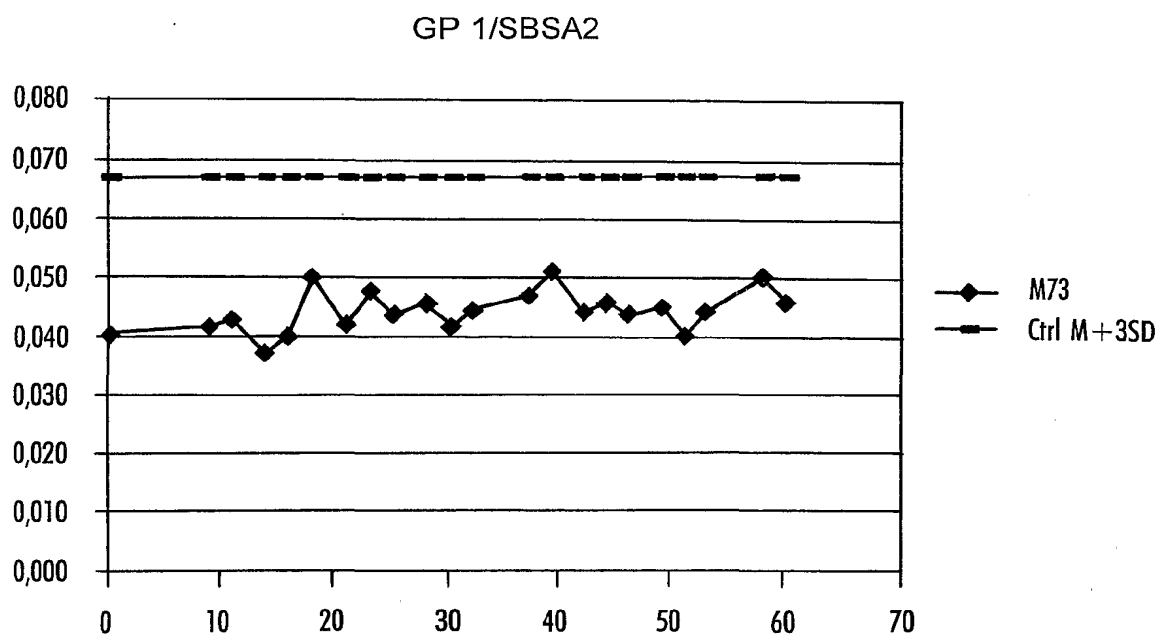


Fig. 7

8/11

**Fig. 8a**

9/11

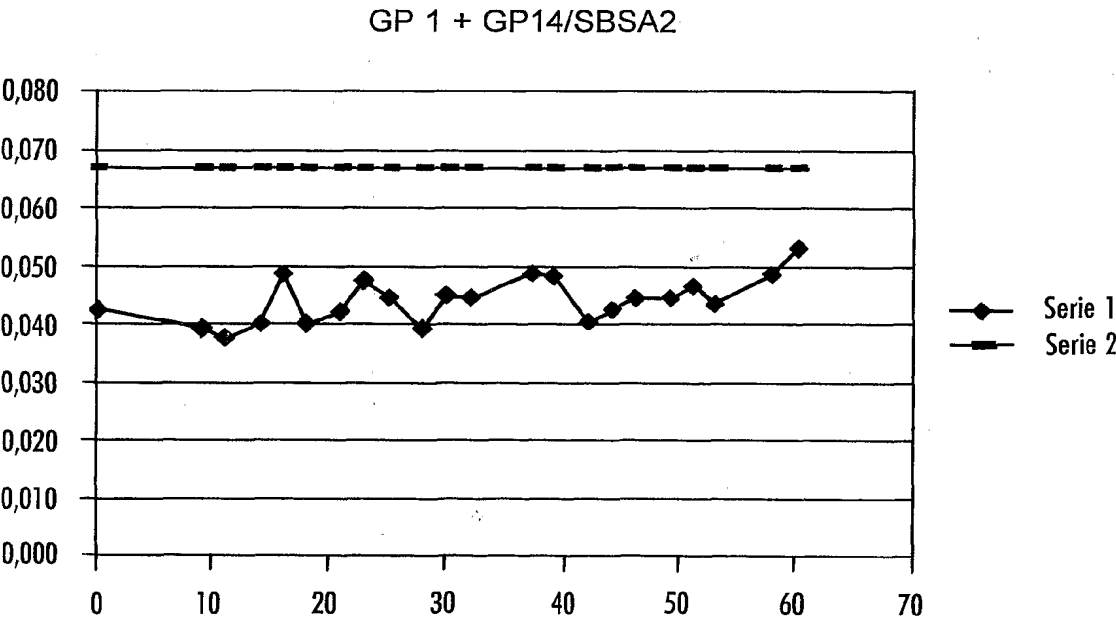
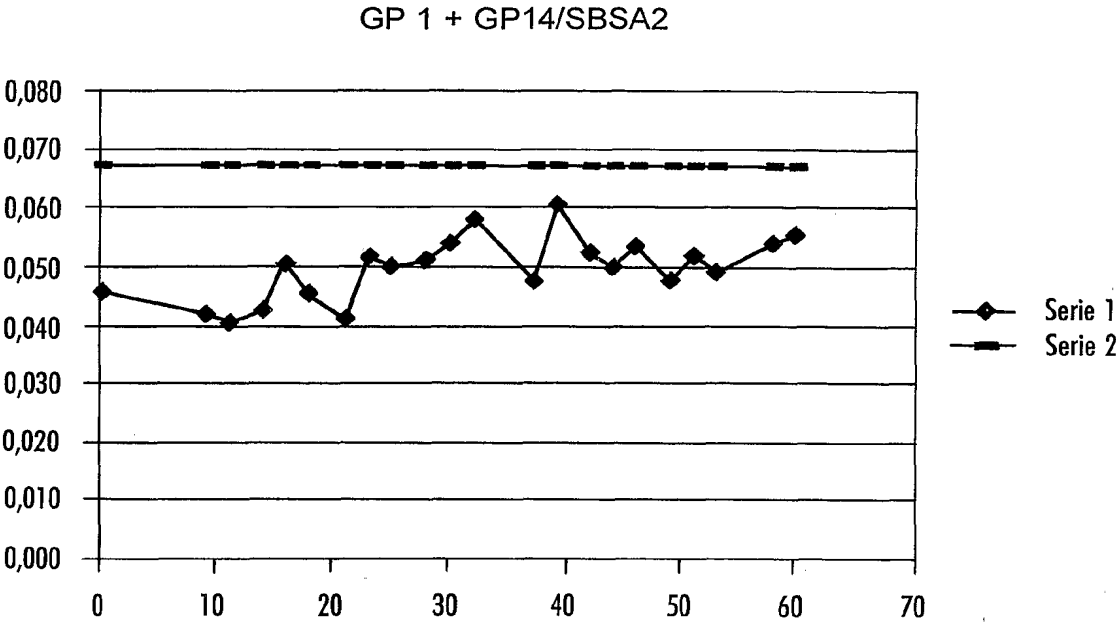


Fig. 8b

10/11

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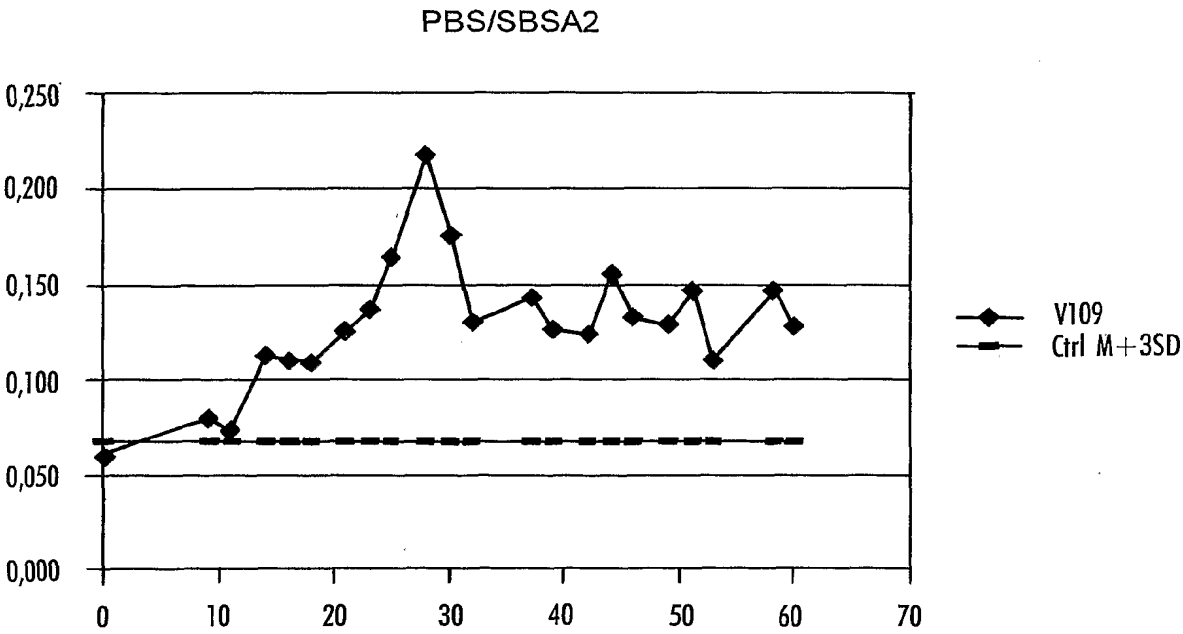
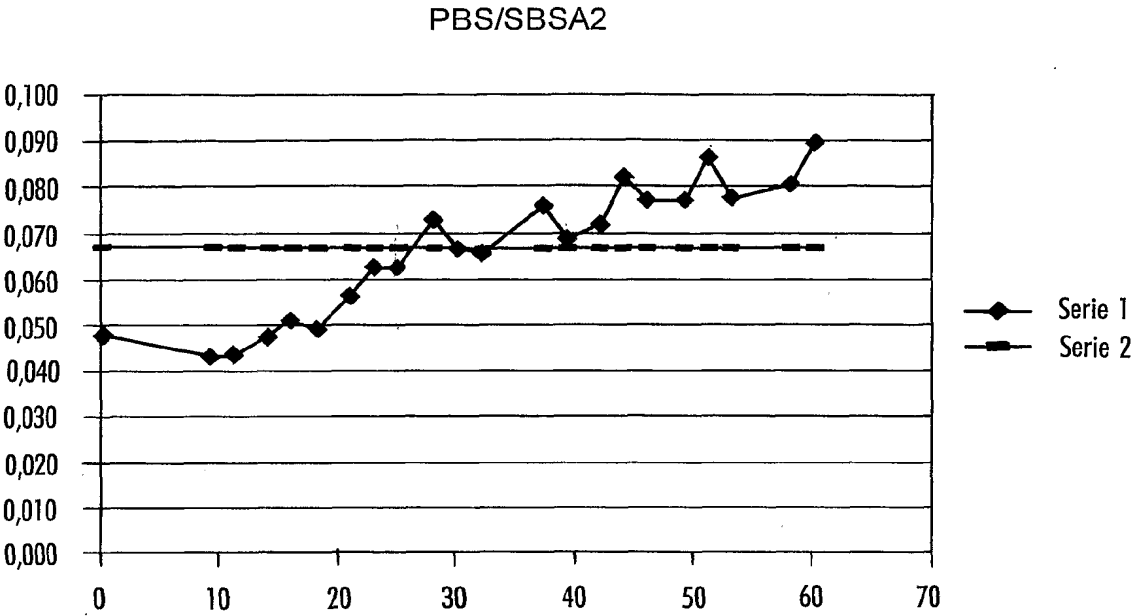


Fig. 8c

11/11

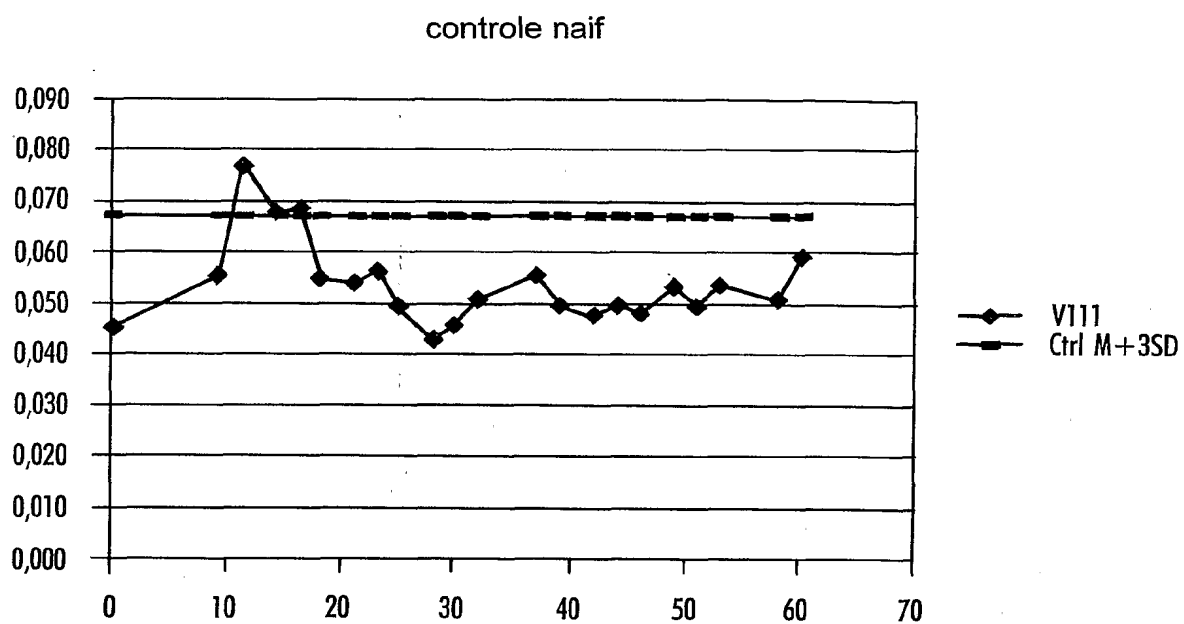


Fig. 8d

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<211> 1787

<212> PRT

<213> K1 parasite clone

<400> 2

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 20 25 30
 Ile Lys Lys Cys His Met Arg Glu Lys Ile Asn Lys Tyr Phe Phe Leu
 35 40 45

Ile	Lys	Ile	Leu	Thr	Cys	Thr	Ile	Leu	Ile	Trp	Ala	Val	Gln	Tyr	Asp	50	55	60
Asn	Asn	Ser	Asp	Ile	Asn	Lys	Ser	Trp	Lys	Lys	Asn	Thr	Tyr	Val	Asp	65	70	75
Lys	Lys	Leu	Asn	Lys	Leu	Phe	Asn	Arg	Ser	Leu	Gly	Glu	Ser	Gln	Val	85	90	95
Asn	Gly	Glu	Leu	Ala	Ser	Glu	Glu	Val	Lys	Glu	Lys	Ile	Leu	Asp	Leu	100	105	110
Leu	Glu	Glu	Gly	Asn	Thr	Leu	Thr	Glu	Ser	Val	Asp	Asp	Asn	Lys	Asn	115	120	125
Leu	Glu	Glu	Ala	Glu	Asp	Ile	Lys	Glu	Asn	Ile	Leu	Leu	Ser	Asn	Ile	130	135	140
Glu	Glu	Pro	Lys	Glu	Asn	Ile	Ile	Asp	Asn	Leu	Leu	Asn	Asn	Ile	Gly	145	150	155
Gln	Asn	Ser	Glu	Lys	Gln	Glu	Ser	Val	Ser	Glu	Asn	Val	Gln	Val	Ser	165	170	175
Asp	Glu	Leu	Phe	Asn	Glu	Leu	Leu	Asn	Ser	Val	Asp	Val	Asn	Gly	Glu	180	185	190
Val	Lys	Glu	Asn	Ile	Leu	Glu	Glu	Ser	Gln	Val	Asn	Asp	Asp	Ile	Phe	195	200	205
Asn	Ser	Leu	Val	Lys	Ser	Val	Gln	Gln	Glu	Gln	Gln	His	Asn	Val	Glu	210	215	220
Glu	Lys	Val	Glu	Glu	Ser	Val	Glu	Glu	Asn	Asp	Glu	Glu	Ser	Val	Glu	225	230	235
Glu	Asn	Val	Glu	Glu	Asn	Val	Glu	Glu	Asn	Asp	Asp	Gly	Ser	Val	Ala	245	250	255
Ser	Ser	Val	Glu	Glu	Ser	Ile	Ala	Ser	Ser	Val	Asp	Glu	Ser	Ile	Asp	260	265	270
Ser	Ser	Ile	Glu	Glu	Asn	Val	Ala	Pro	Thr	Val	Glu	Glu	Ile	Val	Ala	275	280	285
Pro	Ser	Val	Val	Glu	Ser	Val	Ala	Pro	Ser	Val	Glu	Glu	Ser	Val	Glu	290	295	300
Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	305	310	315
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Pro	Thr	Val	Glu	Glu	Ile	Val	Ala	Pro	Ser	Val	Val	Glu	Ser	Val	Ala	355	360	365
Pro	Ser	Val	Glu	Glu	Ser	Val	Glu	Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	370	375	380
Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	385	390	395
Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	405	410	415
Glu	Asn	Val	Glu	Glu	Ile	Val	Ala	Pro	Thr	Val	Glu	Glu	Ile	Val	Ala	420	425	430
Pro	Thr	Val	Glu	Glu	Ile	Val	Ala	Pro	Ser	Val	Val	Glu	Ser	Val	Ala	435	440	445
Pro	Ser	Val	Glu	Glu	Ser	Val	Glu	Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	450	455	460
Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	465	470	475
Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	485	490	495
Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	500	505	510
Glu	Asn	Val	Glu	Glu	Ile	Val	Ala	Pro	Thr	Val	Glu	Glu	Ile	Val	Ala	515	520	525
Pro	Thr	Val	Glu	Glu	Ile	Val	Ala	Pro	Ser	Val	Val	Glu	Ser	Val	Ala	530	535	540
Pro	Ser	Val	Glu	Glu	Ser	Val	Glu	Glu	Asn	Val	Glu	Glu	Ser	Val	Ala	545	550	555
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Glu Asn Val	Glu	Glu	Ser Val	Ala	Glu Asn Val	Glu Glu Ser	Val Ala	
	565				570		575	
Glu Asn Val	Glu	Glu	Ser Val	Ala	Glu Asn Val	Glu Glu Ile	Val Ala	
	580				585		590	
Pro Thr Val	Glu	Glu	Ile Val	Ala	Pro Thr Val	Glu Glu Ile	Val Ala	
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Pro Ser Val	Val	Glu	Ser Val	Ala	Pro Ser Val	Glu Glu Ser	Val Glu	
	610			615		620		
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	625			630		635		640
Glu Asn Val	Glu	Glu	Ser Val	Ala	Glu Asn Val	Glu Glu Ile	Val Ala	
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Pro Thr Val	Glu	Glu	Ile Val	Ala	Pro Thr Val	Glu Glu Ile	Val Ala	
	660				665		670	
Pro Ser Val	Val	Glu	Ser Val	Ala	Pro Ser Val	Glu Glu Ser	Val Glu	
	675			680		685		
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	690			695		700		
Glu Asn Val	Glu	Glu	Ser Val	Ala	Glu Asn Val	Glu Glu Ser	Val Ala	
	705			710		715		720
Glu Asn Val	Glu	Glu	Ile Val	Ala	Pro Thr Val	Glu Glu Ile	Val Ala	
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Pro Thr Val	Glu	Glu	Ile Val	Ala	Pro Ser Val	Val Glu Ser	Val Ala	
	740				745		750	
Pro Ser Val	Glu	Glu	Ser Val	Glu	Glu Asn Val	Glu Glu Ser	Val Ala	
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Glu Asn Val	Ala	Thr	Asn Leu	Ser	Asp Asn Leu	Leu Ser	Asn Leu	Leu
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Gly Gly Ile	Glu	Thr	Glu	Glu	Ile Lys Asp	Ser Ile	Leu Asn	Glu Ile
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Glu Glu Val	Lys	Glu	Asn Val	Val	Thr Thr Ile	Leu Glu	Asn Val	Glu
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Glu Thr Thr	Ala	Glu	Ser Val	Thr Thr	Phe Ser	Asn Ile	Leu Glu	Glu
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Ile Gln Glu	Asn	Thr	Ile Thr	Asn Asp	Thr Ile	Glu Glu	Lys Leu	Glu
		885			890		895	
Glu Leu His	Glu	Asn	Val Leu	Ser	Ala Ala	Leu Glu	Asn Thr	Gln Ser
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Glu Glu Glu	Lys	Lys	Glu Val	Ile	Asp Val	Ile Glu	Glu Val	Lys Glu
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Glu Val Ala	Thr	Thr	Leu Ile	Glu Thr	Val Glu	Gln Ala	Glu Glu	Lys
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Ser Ala Asn	Thr	Ile	Thr Glu	Ile	Phe Glu	Asn Leu	Glu Glu	Asn Ala
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Val Glu Ser	Asn	Glu	Asn Val	Ala	Glu Asn	Leu Glu	Lys Leu	Asn Glu
		965			970		975	
Thr Val Phe	Asn	Thr	Val Leu	Asp	Lys Val	Glu Glu	Thr Val	Glu Ile
	980				985		990	
Ser Gly Glu	Ser	Leu	Glu Asn	Asn Glu	Met Asp	Lys Ala	Phe Phe	Ser
	995			1000		1005		
Glu Ile Phe	Asp	Asn	Val Lys	Gly Ile	Gln Glu	Asn Leu	Leu Thr	Gly
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Met Phe Arg	Ser	Ile	Glu Thr	Ser Ile	Val Ile	Gln Ser	Glu Glu	Lys
	1025			1030		1035		104
Val Asp Leu	Asn	Glu	Asn Val	Val	Ser Ser	Ile Leu	Asp Asn	Ile Glu
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 Asp Ser Lys Glu Glu Thr Glu Ser Ile Lys Asp Lys Glu Lys Asp Val
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 1235 1240 1245
 Glu Leu Asn Glu Val Glu Ala Asp Leu Ile Lys Asp Met Glu Lys Leu
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 1265 1270 1275 128
 Ala Lys Asp Asp Thr Leu Glu Lys Val Ile Glu Glu Glu His Asp Ile
 1285 1290 1295
 Thr Thr Thr Leu Asp Glu Val Val Glu Leu Lys Asp Val Glu Glu Asp
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 Lys Ile Glu Lys Val Ser Asp Leu Lys Asp Leu Glu Glu Asp Ile Leu
 1315 1320 1325
 Lys Glu Val Lys Glu Ile Lys Glu Leu Glu Ser Glu Ile Leu Glu Asp
 1330 1335 1340
 Tyr Lys Glu Leu Lys Thr Ile Glu Thr Asp Ile Leu Glu Glu Lys Lys
 1345 1350 1355 136
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 Phe Phe Ser Lys Val Lys Asn Phe Val Lys Lys Tyr Lys Val Phe Ala
 1650 1655 1660
 Ala Pro Phe Ile Ser Ala Val Ala Ala Phe Ala Ser Tyr Val Val Gly
 1665 1670 1675 168
 Phe Phe Thr Phe Ser Leu Phe Ser Ser Cys Val Thr Ile Ala Ser Ser
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 Tyr Leu Gln Gln Met Lys Glu Lys Phe Ser Lys Glu Lys Asn Asn Asn
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 Val Ile Glu Val Thr Asn Lys Ala Glu Lys Lys Gly Asn Val Gln Val
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 1765 1770 1775
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 1780 1785

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 <212> DNA
 <213> T9/96 Parasite Clone

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 caacaagaac aacaacacaa tgttgaagaa aaagttagaag aaagtgtaga agaaaatgac 180
 gaagaaagtg tagaagaaaa tgtagaagaa aatgtagaag aaaatgacga cggaagtgtgta 240
 gcctcaagtg ttgaagaaag tatagcttca agtgttgatg aaagtataga ttcaagtatt 300
 gaagaaaatg tagctccaac tgttgaagaa atcgtagctc caactgttga agaaattgtgta 360
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 gaagaaagtg tagctgaaaa tgttgaagaa agtgtagctg aaaatgttga agaaatcgta 480
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 gaagaaagtg tagctgaaaa tgttgaagaa agtgtagctg aaaatgttga agaaagtgtgta 600
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 gttccaagtg ttgaagaaag tgtagctcca agtgttgaag aaagtgtagc tgaaaatgtt 780
 gaagaaagtg tagctgaaaa tgttgaagaa agtgtagctg aaaatgttga agaaagtgtgta 840
 gctgaaaatg ttgaagaaag tgttagctgaa aatgttgaag aaatcgtagc tccaagtgtt 900
 gaagaaatcg tagctccaac tgttgaagaa agtgttgctg aaaacgttgc aacaaattta 960
 tcagacaatc ttttaagtaa tttattaggt ggtatcgaaa ctgaggaaat aaaggacagt 1020
 atattaaatg agatagaaga agtaaaaagaa aatgtagtca ccacaatact agaaaaagta 1080
 gaagaaacta cagctgaaag tgtaactact ttagtaata tattagagga gatacaagaa 1140
 aatactatta ctaatgatac tatagaggaa aaattagaag aactccacga aatgtatta 1200
 agtgccgctt tagaaaatac ccaaagtga gaggaagaaga aagaagtaat agatgtaatt 1260
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 aatgaaaaag ttgcagagaa tttagagaaa ttaaacgaaa ctgtatttaa tactgtatta 1440
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<211> 570
 <212> PRT
 <213> T9/96 Parasite Clone

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 Phe Asn Ser Leu Val Lys Ser Val Gln Gln Glu Gln Gln His Asn Val
 35 40 45
 Glu Glu Lys Val Glu Glu Ser Val Glu Glu Asn Asp Glu Glu Ser Val
 50 55 60
 Glu Glu Asn Val Glu Glu Asn Val Glu Glu Asn Asp Asp Gly Ser Val
 65 70 75 80
 Ala Ser Ser Val Glu Glu Ser Ile Ala Ser Ser Val Asp Glu Ser Ile
 85 90 95
 Asp Ser Ser Ile Glu Glu Asn Val Ala Pro Thr Val Glu Glu Ile Val
 100 105 110
 Ala Pro Thr Val Glu Glu Ile Val Ala Pro Ser Val Val Glu Ser Val
 115 120 125
 Ala Pro Ser Val Glu Glu Ser Val Ala Pro Ser Val Glu Glu Ser Val
 130 135 140
 Ala Glu Asn Val Glu Glu Ser Val Ala Glu Asn Val Glu Glu Ile Val
 145 150 155 160
 Ala Pro Ser Val Glu Glu Ser Val Ala Glu Asn Val Glu Glu Ser Val
 165 170 175
 Ala Glu Asn Val Glu Glu Ser Val Ala Glu Asn Val Glu Glu Ser Val
 180 185 190
 Ala Glu Asn Val Glu Glu Ser Val Ala Glu Asn Val Glu Glu Ile Val
 195 200 205
 Ala Pro Thr Val Glu Glu Ser Val Ala Pro Thr Val Glu Glu Ile Val
 210 215 220
 Ala Pro Thr Val Glu Glu Ser Val Ala Pro Thr Val Glu Glu Ile Val
 225 230 235 240
 Val Pro Ser Val Glu Glu Ser Val Ala Pro Ser Val Glu Glu Ser Val
 245 250 255
 Ala Glu Asn Val Glu Glu Ser Val Ala Glu Asn Val Glu Glu Ser Val
 260 265 270
 Ala Glu Asn Val Glu Glu Ser Val Ala Glu Asn Val Glu Glu Ser Val
 275 280 285
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 290 295 300
 Ala Pro Thr Val Glu Glu Ser Val Ala Glu Asn Val Ala Thr Asn Leu
 305 310 315 320
 Ser Asp Asn Leu Leu Ser Asn Leu Leu Gly Gly Ile Glu Thr Glu Glu
 325 330 335
 Ile Lys Asp Ser Ile Leu Asn Glu Ile Glu Glu Val Lys Glu Asn Val
 340 345 350
 Val Thr Thr Ile Leu Glu Lys Val Glu Glu Thr Thr Ala Glu Ser Val
 355 360 365
 Thr Thr Phe Ser Asn Ile Leu Glu Glu Ile Gln Glu Asn Thr Ile Thr
 370 375 380
 Asn Asp Thr Ile Glu Glu Lys Leu Glu Glu Leu His Glu Asn Val Leu
 385 390 395 400
 Ser Ala Ala Leu Glu Asn Thr Gln Ser Glu Glu Glu Lys Lys Glu Val
 405 410 415
 Ile Asp Val Ile Glu Glu Val Lys Glu Glu Val Ala Thr Thr Leu Ile
 420 425 430
 Glu Thr Val Glu Gln Ala Glu Glu Ser Glu Ser Thr Ile Thr Glu
 435 440 445
 Ile Phe Glu Asn Leu Glu Glu Asn Ala Val Glu Ser Asn Glu Lys Val
 450 455 460
 Ala Glu Asn Leu Glu Lys Leu Asn Glu Thr Val Phe Asn Thr Val Leu

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465          470          475          480
Asp Lys Val Glu Glu Thr Val Glu Ile Ser Gly Glu Ser Leu Glu Asn
          485          490          495
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          500          505          510
Gly Ile Gln Glu Asn Leu Leu Thr Gly Met Phe Arg Ser Ile Glu Thr
          515          520          525
Ser Ile Val Ile Gln Ser Glu Glu Lys Val Asp Leu Asn Glu Asn Val
          530          535          540
Val Ser Ser Ile Leu Asp Asn Ile Glu Asn Met Lys Glu Gly Leu Leu
545          550          555          560
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<210> 5
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<213> Artificial Sequence

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<220>
<223> Synthetic peptide

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<400> 5
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Leu Asn Asn Ile
20

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<210> 6
<211> 25
<212> PRT
<213> Artificial Sequence

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<220>
<223> synthetic peptide

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<400> 6
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Val Lys Glu Asn Ile Leu Glu Glu Ser
20          25

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<210> 7
<211> 26
<212> PRT
<213> Artificial Sequence

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<220>
<223> synthetic peptide

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<400> 7
Leu Glu Glu Ser Gln Val Asn Asp Asp Ile Phe Asn Ser Leu Val Lys
1          5          10          15
Ser Val Gln Gln Glu Gln Gln His Asn Val
20          25

```

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<210> 8
<211> 28
<212> PRT
<213> Artificial Sequence

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<220>
<223> synthetic peptide

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<400> 8

Val Glu Ser Val Ala Pro Ser Val Glu Glu Ser Val Ala Pro Ser Val
 1 5 10 15
 Glu Glu Ser Val Ala Glu Asn Val Glu Glu Ser Val
 20 25

<210> 9

<211> 123

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic peptide

<400> 9

Leu Ala Ser Glu Glu Val Lys Glu Lys Ile Leu Asp Leu Leu Glu Glu
 1 5 10 15
 Gly Asn Thr Leu Thr Glu Ser Val Asp Asp Asn Lys Asn Leu Glu Glu
 20 25 30
 Ala Glu Asp Ile Lys Glu Asn Ile Leu Leu Ser Asn Ile Glu Glu Pro
 35 40 45
 Lys Glu Asn Ile Ile Asp Asn Leu Leu Asn Asn Ile Gly Gln Asn Ser
 50 55 60
 Glu Lys Gln Glu Ser Val Ser Glu Asn Val Gln Val Ser Asp Glu Leu
 65 70 75 80
 Phe Asn Glu Leu Leu Asn Ser Val Asp Val Asn Gly Glu Val Lys Glu
 85 90 95
 Asn Ile Leu Glu Glu Ser Gln Val Asn Asp Asp Ile Phe Asn Ser Leu
 100 105 110
 Val Lys Ser Val Gln Gln Glu Gln Gln His Asn
 115 120

<210> 10

<211> 96

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic peptide

<400> 10

Glu Ser Val Ala Glu Asn Val Glu Glu Ser Val Ala Glu Asn Val Glu
 1 5 10 15
 Glu Ile Val Ala Pro Thr Val Glu Glu Ile Val Ala Pro Thr Val Glu
 20 25 30
 Glu Ile Val Ala Pro Ser Val Val Glu Ser Val Ala Pro Ser Val Glu
 35 40 45
 Glu Ser Val Glu Glu Asn Val Glu Glu Ser Val Ala Glu Asn Val Glu
 50 55 60
 Glu Ser Val Ala Glu Asn Val Glu Glu Ser Val Ala Glu Asn Val Glu
 65 70 75 80
 Glu Ser Val Ala Glu Asn Val Glu Glu Ile Val Ala Pro Thr Val Glu
 85 90 95